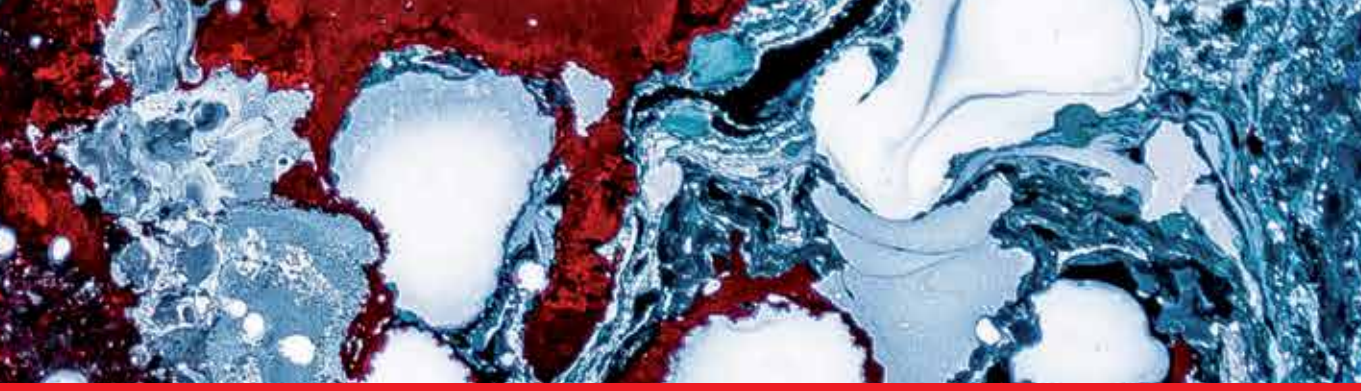

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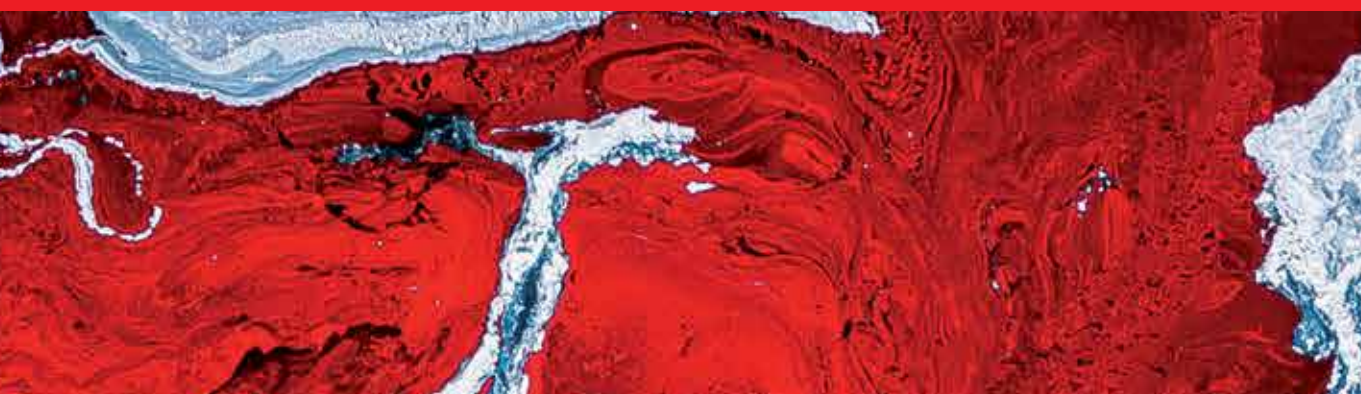

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Meet the editor

Dr. Naofumi Shiomi studied recombinant yeast as a researcher at the Laboratory of Production Technology of Kanena Corporation for 15 years until 1998 and earned his PhD degree in Engineering from Kyoto University. He now works as a professor at the School of Human Sciences of Kobe College in Japan, where he teaches biotechnology and life science in his "Applied Life Science" laboratory. He has studied bioremediation and biomedical science for 23 years at Kobe College and has published more than 50 papers and several books. His recent research has focused on the prevention of obesity and aging.

Contents

Preface

Gene manipulation and polymerase chain reaction technologies, which were born in the late 1870s, drastically changed the world of biotechnology. A substantial number of genes have been cloned from various species, and many genome sequences have been determined using these technologies. Moreover, the emergence of cloning technology and the discovery of embryonic stem (ES) cells drastically changed life science research. In the last several decades, the pathology of many diseases was elucidated by testing transgenic animals. The discovery of ES cells leads to the discovery of induced pluripotent stem (iPS) cells, and regenerative medicine using iPS cells is being used in several clinical trials at present. Biotechnology in life science continues to progress on a day-to-day basis.

With great expectations, the progress of biotechnology has imparted novel and important roles to biochemical engineers. Previously, the main role of biochemical engineers had been to design bioreactors that produced industrially important compounds under optimal conditions. Examples of these new roles for biochemical engineers include application of gene manipulation technology to bioremediation and production of bioenergy and novel chemicals. Moreover, the role has expanded to the medical field where tissue engineering, drug delivery, and therapy have grown drastically.

Based on this background, this book deals with current topics in biochemical engineering. The chapters of this book discuss research that has introduced artificial enzymes, kinetic models in bioprocessing, a small-scale production process, and production of energy with microbial fuel. These chapters offer novel ideas for the production of effective compounds and energy. Moreover, other research has introduced the production technology of stem cells and biomedical processes using nanoshells and extracellular vesicles, which will give important information for regenerative medicine and therapy.

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Chapter 1

Introductory Chapter: Artificial Enzyme Produced by Directed Evolution Technology

Naofumi Shiomi

1. Introduction

Until the middle of 1970s, the roles of biochemical engineers had been to design effective processes for production of industrially important proteins and to control bioreactors under optimal conditions. Many microorganisms were isolated from soils, rivers, and seawater, and their genes were randomly disrupted by mutagenic agents such as 1-methyl-3-nitro-1-nitrosoguanidine to enhance the productivity, which was the best procedure of breeding for a long time. Gene manipulation technology born in the late 1870s was a groundbreaking invention, and the technology drastically changed the world of biotechnology. Polymerase chain reaction (PCR) method and genome analysis using high-performance DNA sequencers, which were born after gene manipulation technology, enhanced the drastic progress. Recently, a huge number of genes have been cloned from isolated microorganisms, and many excellent vector plasmids and promoters have been developed.

Recently, the interest of biochemical engineers shifted from breeding using gene manipulation technology to using the discovered novel biocatalysts and the "directed evolution" technology. The idea of directed evolution was proposed by Arnold et al. They proposed that artificial evolution, which is similar to the natural evolution that slowly occurs in the nature world, can be performed at rapid speed using biotechnology. Based on this concept, many studies have been conducted and have become an important target of biochemical engineers. In the future, such artificial proteins will introduce a next wave in the world of biotechnology [1]. Based on this background, I introduce in this chapter the recent advancements in artificial enzymes. There are other important targets for biochemical engineers, and therefore, I recommend referring to other chapters of this book by other authors.

2. Construction of artificial proteins

The scheme of the screening process of an artificial protein or enzyme using directed evolution technology is shown in **Figure 1**. The screening process in a single cycle is composed of three steps: (1) construction of DNA (or RNA) variants, (2) construction of display library composed of both DNA (or RNA) variants and protein molecules, and (3) screening of target DNA variants. Directed evolution is completed by several repeats of these steps to obtain an optimal enzyme (or protein). Each step is discussed in the following sections.

2.1 Computational design of artificial protein

Structural design by computational simulation provides important information to determine the optimal protein. There are many types of folds and motifs, the elements that compose the tertiary structure. Because of these elements, two proteins can have similar tertiary structures when they have more than 30% homology in their amino acid sequences, and the structural similarity is enhanced in proportion to increasing homology, according to the DBAli database. Moreover, homology models such as consensus protein design [2], phylogeny-based design [3], and design combined with simulation by molecular dynamics [4] have been recently developed. Therefore, the tertiary structure of an unknown protein can be predicted using a known homologous protein.

In the twentieth century, precise prediction of the tertiary structure of proteins had been difficult due to insufficient operation speed of computers. However, the operation speed of computers has drastically improved in the last 10 years. Using a recently developed supercomputer such as Kei computer [5], complicated structural analysis of proteins can be completed in a considerably shorter time. In addition to the progress in computer technology, high-performance DNA sequencers have also entered the next generation. HiSeq2000 DNA sequencer, which equips many flow cells having a huge number of template DNA, can determine giga bases of DNA sequences at one run. Data on genomes of many organisms have been added to data libraries at high speed owing to breakthrough in the DNA sequencing technology. Data on protein structure also have been enriched. The Protein Data Bank (PDB) is a data bank which includes experimental data such as X-ray crystal structural analysis and NMR data, which are important for prediction of 3D structures of proteins [6]. The data in PDB has rapidly increased in the past several years. Accordingly, computational analysis can now yield sufficiently correct 3D structures of proteins and is one of the most prominent tools to design artificial proteins.

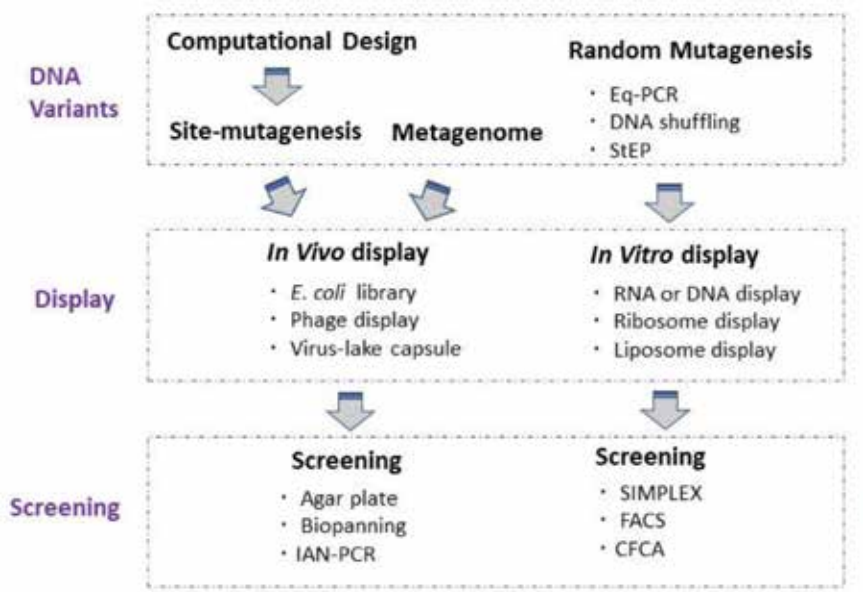

Figure 1.
Scheme of the screening process for artificial protein or enzyme.

2.2 Site mutagenesis and random mutagenesis

Mutation at the protein parts determined by computational structure simulation is generally performed by site mutagenesis or saturation mutagenesis method (site-direct mutagenesis at hotspots) [7]. For instance, activity of α-amylase was enhanced 16.7-fold using site-directed mutagenesis [7], and thermostability of transglutaminase was enhanced twelvefold at 60°C by saturation mutagenesis [8]. Truncation was also used for the mutation based on computationally designed results [9]. Enzymes include unnecessary domains, and characteristics of proteins are often improved by truncation. For example, the enzymes truncated at the N-terminus and C-terminus selected using a randomly truncated library showed high thermostability [10] and activity [11]. The most important advantage of site-direct mutagenesis is that the improved protein can be rapidly selected, because library size required for screening is much smaller than that in random mutagenesis.

Random mutagenesis is also a powerful tool to obtain novel enzymes [9], and several procedures for random mutagenesis such as error-prone PCR (ep-PCR), DNA shuffling, and staggered extension process (StEP) were proposed. In ep-PCR, random mutagenesis is introduced by PCR using error-prone polymerase under high concentration of Mn^{2+} and/or nonuninform dNTP concentration [12]. For example, activity of the subtilis E variant obtained by the ep-PCR method was 256 times higher in 60% DMF solution [13]. In DNA shuffling, many DNA variants containing mutations at different sites are digested with deoxyribonuclease, and PCR is performed using these fragments as template DNAs [14]. StEP is an improved method of DNA shuffling [15]. In this method, random mutation is introduced by repeated short DNA extension times in PCR with many DNA variants as template DNA. Random mutagenesis is a powerful tool to create unknown proteins.

2.3 Novel proteins based on mutagenesis analysis

Soils and seawater are abundant resources of microorganisms. According to the study on the sequences of DNAs of 16S rRNA of soil microorganisms [16], the ratio of microorganism which cannot grow under normal culture conditions to total microorganisms was 99%, suggesting that prominent biocatalysis may be present among microorganisms which cannot be cultured in media.

Metagenome is all genomes contained in the soil or water sample, and metagenomics which deals with metagenome is the other powerful tool to obtain proteins that we cannot even imagine [17, 18]. The procedure to obtain metagenome is as follows: DNAs of all microorganisms obtained from a target portion are purified without culture, digested to appropriated length with a restriction enzyme, and metagenomic library is constructed by combining the digested DNAs with adequate vectors. Metagenomic library of microorganisms living in special and harsh environment is especially useful because the enzymes have extremely prominent activity even though most of them are hard to culture under normal condition. Screening is recently performed to the environment such as hypersaline soda lake sediments [19] and hot environment [20, 21], and their metagenome data is accumulating day by day.

2.4 Display and screening to obtain important artificial proteins

2.4.1 In vitro display

Construction of display libraries and screening are the most important step in directed evolution technology [22]. In vitro and in vivo displays were proposed

[23, 24], and in vitro displays use a cell-free system. Cell-free systems contain components for protein synthesis such as ribosome, energy regeneration substrates, amino acids, and cofactors. Crude extract purified from wheat germ or insect cells [25, 26] and PURE system [27], a mixture of components separately purified from *Escherichia coli* cells, can be used as cell-free system. Protein can form normal tertiary structure by the addition of adequate chaperones.

In vitro display is a protein which is connected to a DNA or RNA variant coding the protein, and various types of this display were proposed [7]. In RNA display, RNA variants combined with puromycin via linker molecules at 3′ terminal of mRNA are used. Puromycin stops the protein synthesis by combining to C-terminus of protein; the protein combined with the mRNA is produced. In ribosome display, RNA variants of which stop codon is removed are used. The ribosome cannot be demolished because of loss of the stop codon; the complex composed of mRNA, protein, and ribosome is produced. In DNA display, the biotin-labeled DNA variant which codes streptavidin gene at the terminal of the gene is used, and protein is synthesized in W/O emulsion. The protein tagged by streptavidin combines with biotin-labeled DNA. Liposome display, a single vesicle liposome containing the cell-free synthesis system, is used. Advantage of liposome display is that membrane protein can be displayed at surface of lipid bilayer.

Target proteins must be screened from displays. In case of screening based on coupling constant, selection of protein is performed based on strength of binding with a target molecule immobilized on a plate or micro-beads. In the case of screening based on enzyme activity, the SIMPLEX method using microplate [28] can be used for the selection of DNA, RNA, and ribosome displays. Display using emulsion [29] and liposome display [30] is more useful for high-throughput screening because FACS or confocal fluorescence coincidence analysis (CFCA) [31] can be used. The advantage of in vitro display using a cell-free system is that construction of library and automation at microscale are easy.

2.4.2 In vivo displays

In vivo system using *E. coli* cells is also used for screening of proteins. The most general procedure is construction of a gene library of *E. coli* transformants: Plasmids containing DNA variants are transformed into *E. coli* cells. Phage display and display using virus-like molecules were also proposed. Phage display, which was developed to display V-region of antibody, can display the target protein at the surface of the phage by the fusion with coat protein of bacteriophage. Relatively large proteins can be displayed by the fusion with N-terminus of g3p protein of M13 phage or C-terminus of g10 protein of T7 phage [32]. Recently, several groups developed display using nucleocapsids as artificial virus [33, 34]. Non-viral cage of lumazine synthase (AaLS) was spontaneously formed, and mRNA could be contained in the capsule.

Selection method of target protein in in vivo displays is as follows: In case of the selection based on bonding strength, protein can be selected with phage display [32]. Biopanning is generally used for the selection from phage display: Phage displays connecting with immobilized molecules are collected and are transformed into *E. coli* cells. In the case of the selection based on enzyme activity, agar plate containing screening medium, which is the general method for screening of microorganisms, is used in the selection of *E. coli* transformant library. Besides this method, a method such as IAN-PCR method, which is based on amino acid sequence, is proposed. The procedure using *E. coli* library is not adapted to select from a huge size of library because the screening speed is very low, but this screening method is adapted to select from small-scale library such as site-mutated library combined with computer simulation or metagenome library.

3. Targets of directed evolution technology

Directed evolution is a trend in twenty-first century, and interesting study subjects have been proposed [35]. One of the interesting study subjects is, of cause, development of super-proteins which are not present in the nature world and can catalyze novel reaction, and the study on Kemp elimination reaction is its pioneer. Natural enzyme catalyzing the Kemp elimination reaction had not been discovered. Röthlisberger et al. produced the eight novel enzymes catalyzing Kemp elimination reaction by using computational design and site mutagenesis [36]. Similarly, Hilvert et al. conducted the site-directed mutagenesis and screened by using droplet-based microfluidic screening platform. The obtained artificial aldolases showed high activity although the original protein showed few activities [37]. Tryptophan synthetase and cytochrome P450 variants obtained by directed evolution could catalyze novel reactions [38]. In addition to directed evolution, metagenomics is a powerful tool to discover such unknown enzymes [39, 40]. For instance, more than unknown 300 nitrilases were discovered from the metagenome library, although nitrilase discovered during many years of study was only less than 20 [41].

Directed evolution technology can be also applied to improvement of metabolic pathways [42]. For example, 1-propanol is expected as a biofuel, but adequate producing strains have not been discovered. Thus, Atsumi et al. produced *E. coli* recombinant by expressing a series of genes for 1-propanol production, and improvement of citramalate synthase gene was conducted by using error-prone PCR and DNA shuffling. The productivities of 1-propanol and 1-butanol in the improved strain were enhanced [43]. Arnold et al. improved alcohol dehydrogenase and ketol-acid reducto-isomerase to enhance the productivity of 1-propanol by directed evolution. The obtained enzymes could use NADH instead of NADPH as a coenzyme [44]. Otherwise, directed evolution technology is applied to regulatory genes for improvement of metabolic pathway. Promoter [45], operon connection [46], and enhancer sequences [47] were improved by the method.

The other interesting study subject is to understand the evolution of life. One approach to the subject is to know which genome length is required for a micro-organism. Gibson et al. constructed artificial genome DNA which only includes chemically synthesized and introduced the genome into microorganisms in which the genome was previously removed [48]. Another research group tried to construct minimal bacterial genome; the constructed genome JCVI-syn3.0 was only 531 kb (473 genes) [49], and the obtained microorganism could grow and showed several characteristics of the microorganism.

The other approach to understand evolution of life is to generate artificial cells using a cell-free system. Szostak et al. randomly synthesized RNAs (1015 of 90 mer) and created a ribozyme having sufficient RNA ligase activity using error-prone PCR with only 10 rounds of repeats [50]. Noireaux et al. constructed cell-sized synthetic vesicle (artificial cells) containing components for translation and transduction [51]. Yomo et al. also produced novel artificial cells, which can progress artificial evolution of RNAs by themselves [52]. Induction of unnatural compounds into cells is another approach. Unnatural basic pairs and more than 100 unnatural amino acids were synthesized, and they were site-specifically introduced into proteins [53]. Accordingly, evolutional RNA engineering may impart validity to the hypothesis of RNA word in the future.

4. Conclusion

Enzymes are useful for industrial chemical reactions, and the role of biochemical engineers was to study the industrial use of enzymes. However, most of the enzymes

that occur in nature do not have sufficient activity for industrial use, and the bioreactors were not as successful as expected. Directed evolution technology is proposed to overcome these problems. Artificial enzymes have been drastically produced by exploiting the direct evolution technology. The technology may be advanced by using editing tools such as TALEN and CRISPR/Cas9. Artificial microorganisms whose genomes are directly improved using CRISPR/Cas9 may be more suitable than recombinant *E. coli* for use as bioreactors. As such, biochemical engineers will play more important roles in the development of bioreactors in the future.

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Chapter 2

Fermentation: Metabolism, Kinetic Models, and Bioprocessing

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Abstract

Biochemical and metabolic interpretation of microbial growth is an important topic in bioreactor design. We intend to address valuable information about the relation of critical operation variables and the simulation of bioprocesses with unstructured and structured kinetic models. Process parameters such as nutrient supply, pH, dissolved oxygen, and metabolic end-products directly impact the physiology and metabolism of microorganisms. Changes in the membrane as well as cell viability are of interest since protein expression and maturation in prokaryota are directly related to membrane integrity. This chapter intends to deliver an insight of different alternatives in kinetic modeling.

Keywords: metabolism, unstructured kinetic models, black box models, gray box models

1. Introduction

Bacteria are the dominant form of life spread across the whole planet. Their biochemistry machinery is well adapted to scarcity conditions; also, they can biosynthesize complex molecules in various environmental conditions. For this reason, the growth and proliferation of bacteria in controlled environments represent an interest of biochemical engineers, microbiologists, and cell-growth enthusiasts since they allow bioprocess simulation and control scheme design. Substrate transformations into cell biomass, organic molecules, therapeutic proteins, biofuels, enzymes, and food additives are of attention since application to actual fields and laboratory experiments are very difficult to scale-up to industrial level with strict and complete control of key variables determined as an ideal process [1]. It is known that the complexity in a mathematical model may increase with the inclusion of environmental conditions such as multisubstrate consumption and product formation, pH change during fermentation, variable temperature, rheological changes in culture media, multiphasic environmental variability, and nonideality of mixing and stirring [2]. The kinetic model had been evolved from simple exponential growth to complex mathematical expressions to predict heterogeneity in single cells, describe multiple reactions, explain internal control mechanisms, and even predict genetic variability between bacterial populations [3]. However, despite the efforts to represent the progress of biological reactions in microbial cultures, the actual application of the model in real production processes is impractical due to a significant amount of information fed to the model [4].

Many of the kinetic growth models base their structure on and take information from empirical observations through experimental data. The white box models (WBMs) use information from mass balances in a single stoichiometric equation where inputs, outputs, and the conversion from substrates to products are followed [5]. Despite effectiveness and advanced reaction representation in WBMs, the representation of the reaction advance degree, some information on metabolic flux analysis (MFA) can be obtained. Models based on detailed MFA can be used to define optimal operation conditions based on biochemical pathways. It has been established that kinetic models of biological reactions are more complicated than "common" chemical reaction models. Microbial growth models require specialized knowledge of rapid changes of environmental conditions, stoichiometric individual reactions, and the appearance of new steady states in different culture stages [6]. In many cases, mechanistic models, based on first principles, are ineffective because of metabolic complexity of microorganisms.

In this sense, complex microbial consortium behavior and culture media with different types of substrates are difficult to model. Nonmechanistic models, or black box models (BBMs), or a combination between mechanistic and nonmechanistic models, or gray box models (GBMs), are more suitable to describe them. Kinetic parameter fitting for WBMs requires experimental measurements of multiple variables, and frequently, model validation may be impractical. BBMs and GBMs constitute alternatives which describe the general dynamic behavior of bioreactors, without requiring many experimental measurements of the system. These models do not offer mechanistic information about metabolic phenomenology present in the system, but they can optimize and control without it. Then, models can be classified based on the mathematical formulation of the system (**Figure 1**). These are classified into mechanistic, empirical, and fermentation models. A mechanistic model is based on deterministic principles. On the other hand, empirical models represent input-output relations without the knowledge of a mechanism. Fermentation process models are usually represented with a combination of both, mechanistic and empirical models.

An important characteristic of modeling is the assumption of homogeneous or heterogeneous conditions. In this sense, a homogeneous system is related to a single continuous phase. In most cases, bioreactors are described as single liquid phases. However, if the biofilm is included in the study, a solid or semisolid phase needs to be considered in the model. On the other hand, heterogeneous systems are related to the description of two or more continuous phases and the interactions between them. Complex heterogeneous systems can be described as multiple phases: liquid,

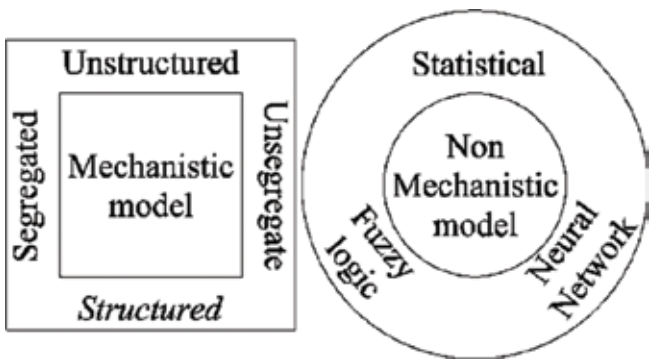

Figure 1.
Classification of models as mechanistic and nonmechanistic.

solid or semisolid, and gaseous phases (e.g. solid-state fermentation). Within this classification, parameters in a model can be classified as distributed and nondistributed (lumped). Distributed parameter models assume that operation parameters vary as a function of space. One, two, or three dimensions are considered in the description of key variables as a function of parameter distribution. As a result, the system is described by a set of partial differential equations (PDEs). On the other hand, a lumped model is necessary, and the system can be described by a set of ordinary differential equations (ODEs), since these parameters do not vary as a function of space.

In this chapter, we provide an overview of mechanistic and empirical models for cell population in fermentation processes.

2. Simple and unstructured kinetic growth models

Unstructured kinetic models (UKMs) represent, in a simple global point of view, the metabolic behavior of the biomass cell production. Mainly, mathematical descriptions for microbial growth kinetics in fermentation processes are based on semiempirical observations. From simple experimental data, we can obtain information to represent cellular growth with unstructured kinetic models.

2.1 Unstructured kinetic models for simple systems

To get the most efficient description of a kinetic model, it is essential to be clear about the application purpose. The application determines the complexity level and structure of the model. The correlation among cell growth, substrate consumption and inhibition [7], or description of the substrate profiles within the reactor during expression of extracellular proteins is the central goal of the model process [8]. The description of key variables is the contribution of the model [9]. These representations are expressed as equations in a simple mathematical model. The UKMs, which are unstructured, unsegregated, are based on the monitoring of cell and nutrient concentration and describe the fermentation process as an average of the species under ideal conditions. Also, it describes the cell and its components as a single species in solution. UKMs consider the apparent rate obtained by metabolic processes, which are carried out by microorganisms. These models are based on conservation equations for cell mass, nutrients, metabolites, and species generation/consumption rates. Most of the UKMs can be divided into three terms: rate expressions for cell growth, rate expressions for nutrient uptake, and rate expressions for metabolite production.

In the case of exponential growth phase, which is the simplest representation of microbial growth, nutrient concentration profiles and decrease rate in several cases are not almost considered.

$$r_X = \frac{dX}{dt} = (\mu - k_D) \cdot X \tag{1}$$

$$r_i = \frac{\alpha}{Y_i}\frac{dX}{dt} \tag{2}$$

where r is the reaction rate, X represents biomass, μ is specific growth rate, k_D is the death rate, α is the stoichiometric factor, and Yi is the yield.

The simplest example of multiple reaction models includes substrate consumption for cell maintenance and true yield coefficients (g DCW/g DW) [5]. One of the

most used UKMs is Monod's model [10]. This is one of the simplest models to deal with microbial growth, physiology, and biochemistry. The Monod equation describes the proportional relationship between the specific growth rate and low substrate concentrations (Eq. (3)).

$$\mu = \frac{\mu_{MAX}[S]}{K_S + [S]} \tag{3}$$

where μ_{MAX} is the maximum specific growth rate, [S] is the substrate concentration, and K_S is the saturation constant.

The disadvantage of the model is that the individual entity, regulatory complex, adaptive response to environmental changes, and capacity of cell organelles to generate various products in inherent metabolism cannot be considered. The simplest mathematical models used to estimate microbial growth and substrate consumption are still used for monoclonal antibody production by Chinese hamster ovary (CHO) cells [11, 12]. UKMs can predict specific growth rate in simple systems by calculation of mass balances with independent variables.

2.2 Unstructured kinetic models for a more complicated system

The Monod equation is not able to predict the substrate inhibition effect. Thus, several models including such effects have been developed. For example, Andrew's kinetic equation includes an inhibition function to relate substrate concentration and specific growth rate [13].

$$\mu = \frac{\mu_{MAX}}{1 + K_S/[S] + [S]/K_i} \tag{4}$$

where K_i is the substrate inhibition parameter.

Under the assumption of steady state in continuous operation, substrate concentration is low, and the term $[S]/K_i$ is neglected. Under these conditions, specific growth rate of Andrew's kinetic equation follows Monod equation [13]. Another inhibition function is Aiba's equation for alcoholic fermentation.

$$\mu = \frac{\mu_{MAX}[S]}{K_S + [S]} e^{(-[P]/K_i)} \tag{5}$$

where [P] is the product concentration.

Under the assumption of low product concentration, the term $[P]/K_i \approx 0$, resulting in a simplification to Monod equation [13].

The Monod model assumes that the fermentation culture media has only one limiting substrate. More than one limiting substrate is present and impacts specific growth rate. Thus, the following model considering multiple substrates is proposed [14].

$$\mu = \left(1 + \sum_i^n \frac{[S_{e,i}]}{[S_{e,i}] + K_{e,i}}\right)\left[\prod_j^n \frac{\mu_{\mathrm{MAX},j}[S_j]}{[S_j] + K_{S,j}}\right] \tag{6}$$

where subscript i is the number of each substrate species and e represents the essential substrate.

The limited but accurate information provided by UKMs may help to represent global reactions effectively. In addition to substrate consumption and microbial

growth, fermentations present catabolic inhibition. Therefore, several research groups propose complete UKMs, which include the empirical observation such as variables regarding cells, substrates, and products. Hans and Levenspiel [15] proposed a kinetic model that assumes the existence of inhibitor critical concentrations.

$$\mu = \mu_{MAX}\left[\prod_{i=1}^{h}\left(1-\frac{[I_i]}{[I_i^*]}\right)^{n_i}\right]\left(\frac{[S]}{[S]+K_S\left[\prod_{i=1}^{h}\left(1-\frac{[I_i]}{[I_i^*]}\right)^{m_i}\right]}\right) \tag{7}$$

where [I] is the inhibitor species concentration.

The inhibition function proposed by Levenspiel [16] takes into account the inhibition of ethanol production of alcoholic fermentation modeling, where subscript i corresponds to the substrate or product concentrations. Linear ($n, m = 1$), nonlinear ($n, m > 1$), and fractional ($n, m = 0.5$) applications of these models are possible for fermentation bioreactors (Eq. (7)). An extension of the model was proposed by Luong [7]. He assumes a common mechanism to describe substrate inhibition. Inhibitory factors acting simultaneously could be represented by the following equation:

$$\mu = \mu_{MAX}\left(\frac{[S]}{[S]+K_S}\right)\left(1-\frac{[P]}{[P_{MAX}]}\right)^{n} \tag{8}$$

where [P] is the product species concentration and n is the index of cooperativity between inhibitors.

These models can also explain multiple reactions and include biochemical information of metabolites in the global net effect, making them experimentally accurate. This characteristic is useful for structured and segregated modeling [17]. These models can also describe mixed metabolism [18] and hetero-fermentations [19]. The duality of *Saccharomyces cerevisiae* metabolism, aerobic and anaerobic metabolism, is the best example of multiple reactions. The aerobic growth of the yeast yields biomass by favoring metabolic pathways designed for anabolism and cell division. This metabolism is oxidative in amphibolic reactions. However, at low oxygen concentrations, the yeast metabolism changes from being purely respiratory to partially fermentative. The fermentative pathway mainly leads to ethanol production as a final electron acceptor. Thus, there is a limited growth with high ethanol yields in fermentation culture media. Both metabolisms can occur during the growth of *S. cerevisiae* in a wide range of simple carbohydrate fermentations. At high substrate concentrations, there are limitations in respiratory pathways, which lead to an overflow to ethanol production with enhanced fermentative pathways. The simple WBM with overall reactions could not explain in detail the dualism of both fermentative and respiratory metabolisms. Thus, there are two stoichiometric reactions proposed to explain oxidative and fermentative metabolisms [18].

$$\gamma_1 X + \beta_{11} CO_2 - S - \alpha_{12} O_2 = 0 \tag{9}$$

$$\gamma_2 X + \beta_{21} CO_2 + \beta_{22} P - S = 0 \tag{10}$$

where α, β, and γ are stoichiometric coefficients.

This system considers nearly ideal Monod kinetics, no by-product formation, linear specific oxygen consumption rate, and correlation with substrate uptake. If the primary carbon source is glucose (instead of ethanol), glucose can be used

aerobically and anaerobically. Ethanol can be used as a carbon source only aerobically. Then, different sets of linear algebraic equations can be derived concerning carbon, oxygen, and hydrogen balance.

The respiratory quotient (RQ) is often used as an indicator of fermentative processes. When RQ is close to one, there is no fermentative metabolism, whereas if RQ is above one, the fermentative metabolism occurs.

$$RQ = \left|\frac{r_{CO_2}}{r_{O_2}}\right| = \begin{cases} >1, \textit{fermentative metabolism} \\ \approx 1, \textit{non fermentative metabolism} \end{cases} \tag{11}$$

The mechanistic characteristics of an unstructured, unsegregated kinetic model contribute to the knowledge of the complex metabolism of *S. cerevisiae*. Despite giving relevant information of simple metabolic processes with multiple reactions, UKMs cannot give information about complete intracellular oxidative metabolism. An example of the application of these models is explained in subsequent sections.

3. Structured growth kinetics

There are several classifications of mechanistic and statistical models of cell population for bioprocess applications. Two terms are essential for mathematical description of cell populations: segregated and structured models. A structured model is related to cell material description using multiple chemical components. A segregate model is related to the description of individual cells in a heterogeneous population. Additionally, it is possible to combine a structured approach with a segregated approach. Structured kinetic models are introduced in this section.

3.1 Simple structured kinetic models

Structured kinetic models (SKMs) describe changes in cell population. The liquid phase (abiotic phase) usually contains nutrients for cell growth and some extracellular metabolites. The microorganisms suspended in the liquid phase behave as multicomponent systems. SKMs consider the internal structure of cells (e.g. mitochondria), and the description of cell growth and its metabolism is used to assume a more accurate growth rate. The information used is a starting point to generate schemes that represent more accurately the growth of microorganisms and their cellular components. The complexity of the information variables and parameters increases in SKMs with the mathematical representation of cellular growth.

SKMs are generally classified into morphologically structured models, chemically structured models, genetically structured models, and metabolically structured models [20].

Morphologically structured models consider the kinetics of nutrient consumption and product formation. These models consider different cell types as living species in terms of the role that they play in the overall reaction. Chemically structured models consider the effects of chemical species in fermentation kinetics; all viable cells are functionally similar, and all the fermentation rates and transport phenomena parameters are accounted for. Genetically structured models assume molecular mechanism knowledge. The model includes the rate of expression of an operator-regulated gene and kinetic equations for the transcription, translation, and folding processes. Metabolically structured models provide a better understanding of process regulation mechanisms such as feedback regulation. This model is based on the main metabolic pathways and in most cases is included in MFA. In the

presence of metabolite concentration changes, the network structure represents the reaction and metabolite concentration as a matrix array. Then, SKMs can be classified as dynamic and structural [21]. Dynamic models are described as a set of ordinary differential equations (ODEs). Structural models, which are simplified from ODEs, are represented by a set of algebraic equations through two main approaches: MFA and elementary mode analysis (EMA).

4. Nonmechanistic models

The structured and unstructured kinetic models in the previous sections describe, with a high degree of accuracy, the dynamic behavior of microbial growth in bioreactors. These models, associated with material and energy balances, also help to understand the phenomena associated with microbial metabolism, giving clues to the process design and control.

Black box models (BBMs) usually fall into two main categories: statistical models (SMs) and artificial intelligence tools (AITs). SMs use experimental design, response surface analysis, and exploratory data analysis, whereas AITs consider tools such as data mining, artificial networks, and fuzzy logic [22]. Also, several methodologies to combine mechanistic approaches with nonmechanistic modeling strategies have been developed. The hybrid models, which are known as gray box models (GBMs), inherit the advantages of BBMs such as data analysis and can achieve semi-mechanistic description to each metabolic phenomenon. GBMs offer greater estimation accuracy, calibration ease, better extrapolation properties, and more detailed information on the phenomenology of the system [23]. The advantages of GBMs in the application of bioreactor modeling are direct control and optimization. In this section, we will describe some of these nonmechanistic modeling tools and some of their applications, such as the design of soft sensors.

4.1 Neural network models

Artificial neural networks (ANNs) are mathematical models that are devised from the need to characterize biological neural processes. As the system of ANNs imitates the way which is used to interact with each other in brain neuron, ANNs are simple and strong processes to interconnect the elements that transmit and process information through electrical impulses. In ANNs, these simple process elements are also known as neurons, and depending on the complexity of the connection schemes, they can develop the ability to describe the nonlinear behavior of many dynamic systems [24]. ANNs are computational models that aim to achieve mathematical formalizations of the brain structure and functions, which are constantly reformed by learning through experience and extracting knowledge from the same experience. In ANNs, the hierarchical structure similar to that in brain is established, where neurons connect with each other and transmit the response to other neurons. Once the ANN's structure is defined, it is necessary to develop memory form experience (experimental data). In order to introduce this experience, the ANN training algorithm performs a weight (ω) fitting process associated with each neuron, such that the actions introduced (input signals) converge to the reactions produced (output signal) [24]. Although ANNs do not provide a physical interpretation of the phenomena that take place in the system, these models can approximate the dynamic behavior of the system, making them suitable universal approximators [22]. ANNs are defined based on three basic characteristics: their architecture, activation functions, and training algorithm. The architecture deals with the type of interconnections between their processing units or neurons, while

the activation function corresponds to the dynamic characteristics of the neuron transfer functions. The training algorithm refers to the parameter fitting procedure, which provides the learning ability.

Feedforward networks (FNN) represent the simplest network configuration capable of describing the nonlinear behavior of bioreactors. In FFNs, neurons of each layer propagate their information to all neurons in subsequent layers. In each neuron, the input information corresponds to the weighted sum of all the outputs of the previous layer, and the weighting factors, weights and thresholds, are internally fitted for better system description [24].

Another type of ANNs frequently used is recurrent neural networks (RNNs). The structure of RNNs differ from FFNs, in the sense that some of the last layer neuron output signals are fed back as inputs to any previous layer. RNNs could converge to stable system solutions and include the effects of response delays. These characteristics make these models especially useful in the modeling of continuous bioreactors [25].

4.2 Gray box models

BBMs are based on the analysis of data generated to detect correlations, and basic functionalities between the variables and the WBMs are constructed from first principles. A hybrid model category between WBMs and BBMs is GBMs; these models implement a set of tools that combine some of the characteristics of both. Some of these characteristics include properties of process and control design, without losing the ability to explain the phenomena present in the system. Defining a parallel or serial data flow structure allows the integration of both, mechanistic and nonmechanistic information (e.g., **Figure 2**).

Parallel arrays are mainly used when there is a well-defined mechanistic model of the process and are suitable to improve its estimation performance. It is especially useful in cases where dynamic aspects of the system can be decoupled. **Figure 2A** shows a conceptual diagram of parallel interaction where the circle represents WBMs and the square BBMs, and the circle inside the square corresponds to a hybrid model. In the case of serial arrays, BBMs describe just specific terms of the WBMs, such as growth kinetics or transport parameters. **Figure 2B** represents the hybrid model where the BBMs (square) are substituted into the WBMs (circle). Stosch et al. presented a detailed panorama of this model [23].

In the design of bioreactors and their associated controllers, one of the difficulties is the determination of the kinetic model that adequately describes the growth rate of the respective microorganisms. The selection of a kinetic model leads to restrictive models for fixed operating conditions with little extrapolation possibility.

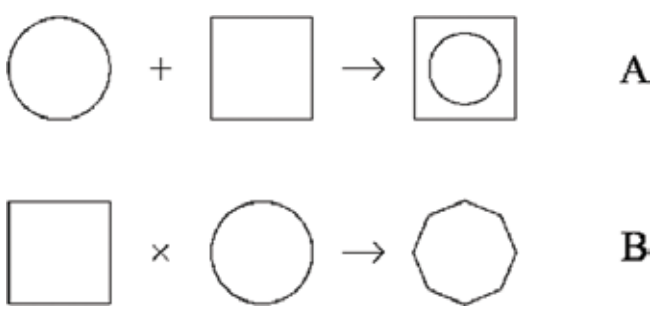

Figure 2.
Classification of models as (A) parallel flow nonmechanistic model and (B) serial flow nonmechanistic model.

GBMs take advantage of the well-known mechanistic information through mass and energy balances to describe specific dynamics of the system and expand its parametric scenario of applicability.

In the determination of bacterial growth kinetic models, GBMs have some advantages compared to BMMs; FFNs are able to describe the system state values, but have important deviations in their estimates, due to their high sensitivity to noise. GBMs combine WBMs with neural network structures, either by feeding the outputs of the ANNs to the state space model, or by backpropagating the estimation error from output layers. Therefore, GBMs offer better forecasting properties and strengthen their performance in the presence of noise [26].

4.3 State observers and soft sensors

Monitoring culture media requires the measurement of variables such as biomass concentration, substrates, dissolved oxygen, carbon dioxide, ammonia, and temperature, among others. These values are used for growth kinetics determination and bioreactor design. However, on multiple occasions, implementation of specific sensors is complicated, and there may be limitations in sensing frequency for variables such as biomass concentration. The implementation of indirect measurement methodologies, such as signal filtering, observer design, and ANNs, allows the estimation of some of these variables, and even the estimation of complex variables such as overall microbial growth rate and the heat flux produced by the system.

In control system theory, a major implementation of state observers is a common complementary strategy. These observers estimate some state variables that cannot be easily measured, either by the absence of suitable sensors, or because of low sampling frequency and high delay times. The main types of observers used for these purposes are those based on the Luenberger scheme, finite-dimensional observers, Bayesian estimators such as Kalman filters, interval observers, observers for fault detection, and even models of artificial intelligence such as ANNs and hybrid models [27].

State observer design requires that the estimated variables are detectable and observable. These states are observable if for a set of specific initial conditions, the internal states of the system are inferred from the knowledge of their outputs. Once its observability is determined, the observer can be designed; the desired type of observer is selected from categories mentioned above. Afterwards, tests of the estimator are carried out by comparing the real values against observer estimates, and in the case of important discrepancies between these values, the observer is adjusted or a different one is selected.

In the case of stirred tank reactors (batch, continuous, or semi-continuous), we may often assume that homogeneous conditions are available, so the system models obtained consist of aggregate parameter systems (ODEs). However, frequently in the case of tubular reactors or solid substrate fermentation systems, homogeneity assumptions are not adequate, so it is necessary to construct using distributed parameter models (PDEs). In the latter case, the design and performance of the main observers, such as Luenberger or Bayesian type, are usually limited, and therefore, it is usual to resort to another type of observer. In such a case, the observers based on a discretized system are substituted for traditional observers [28].

Soft sensors or virtual sensors are used as state observers in specific application. These soft sensors combine several physical measurements with dynamic characteristics to calculate other variables that are not measured.

Soft sensors can not only provide variable information to characterize a system but also facilitate the design of the control schemes.

In bioreactor design, soft sensors can be used to estimate unavailable variables such as biomass. Traditionally, biomass has been traditionally determined by use of a variety of methodologies such as optical density, dry weight, and microbial counts, among others. These techniques present several problems, the most important being the lack of continuous online measurements. To overcome this problem, various strategies have been applied, such as the implementation of low-cost sensors combined with signal processing strategies. For instance, the RGB sensor is used for biomass measurement in microalgae production reactors [29]. This type of sensors uses the intensity of the red, green, and blue (RGB) colors, which correlates with the biomass concentration using dry weight and/or colony formation unit (CFU) information, using the Beer-Lambert law principles. The correlation is described through linear fitting [30]. Additionally, it is possible to compensate background noise by use of ANNs even in the case of nonlinear correlation [31].

Soft sensors can also be applied to nonexplicit system states. These observers can estimate lumped system variables, such as growth rate. As the simplest factor, temperature is commonly used, since it allows estimating system concentrations, due to intrinsic dependence between reaction rates and reaction enthalpy. The heat of reaction, either consumed or dissipated by the system, is one of the implicit system states used for reaction rate determination. The same strategy may also be used to determine microbial growth rates [32].

Microbial growth rates are inherently variable due to their metabolic nature and operation conditions. For example, as fluctuation in substrate concentration occurs in fed-batch bioreactors, the condition of osmotic pressure within cells is modified through the plasma membrane, which may change cellular energetics and the viability of cell division. A suitable strategy for these cases is the design of a substrate consumption rate observer. This kind of observer helps to design a robust control strategy against important fluctuations in maintaining constant substrate concentrations.

The use of observers or soft sensors is an interesting alternative to elucidate approximate values of system states, whether these are explicit or implicit, in cases where online continuous physical measurement is not available. These approximations can be used to design process control schemes that ensure proper functioning.

5. Application examples

There are many practical applications of structured or unstructured kinetic models. In the acetone-butanol-ethanol (ABE) production, the models for the bioprocess have evolved from simple stoichiometric equations to sophisticated and elaborate kinetic models based on metabolic pathways [33, 34], genome-scale metabolic flux modeling [35], system-level modeling [34], and metabolic network [21]. Gordeeva classified mathematical modeling of specific growth rate (dependent or independent on substrate concentration), specific rate of substrate consumption, and specific rate of product formation in batch fermentations [36]. In this study, the states in fermentation are described by a system of three ODEs [36]. Cui reported unstructured lactate formation by enzymatic hydrolysis of sugarcane bagasse, and the model is based on Logistic equations, Luedeking-Piret equations and Luedeking-Piret-like equations [37]. Similarly, Sharma reported an unstructured model to describe growth, substrate utilization, and lactate production by *Lactobacillus plantarum* [38]. On the other hand, the common mathematical descriptions of the fermentation process are based on UKMs. For example, the fermentation of sweet sorghum stalk juice by immobilized *Saccharomyces cerevisiae* is explained by the kinetic parameters of Hinshelwood's model [39]. Another example using the UKM

model is the basic logistic model incorporated with the Luedeking-Piret model (hybrid model) to describe the production of bioethanol from banana and pineapple wastes [40].

Cephalosporium acremonium (ATCC 36225) is one example of the utilization of SKMs where morphological differentiation and catabolite repression are the main aspects of the model approach [41]. SKMs can also effectively represent diauxic growth as well as the monitoring of an intracellular reactant in acetic acid production by *Bacillus licheniformis* [42]. Sansonetti reported a biochemically structured model for ethanol production from ricotta cheese whey by *Kluyveromyces marxianus* [43]. Wang studied a segregated kinetic model in fed-batch culture to represent simultaneous saccharification and co-fermentation (SSCF) for bioethanol production from lignocellulosic raw materials at high substrate concentrations [44]. Another interesting process is the solid-state fermentation. In most proposed models, a set of PDEs is used to describe how intraparticles are diffused or how the growth can be affected by intraparticle diffusion of oxygen, enzymes, hydrolysis products, and other nutrients and the role in the fermentation of other phenomena such as particle shrinkage and spatial microbial biomass distribution [45]. Computational fluid dynamics (CFD) provides information concerning the mixing modeling and design of bioreactors [46]. Another example of CFD is cephalosporin production by *Acremonium chrysogenum;* it was found that the oxygen transfer rate (OTR) directly affects fermentation performance with different impeller combinations [47]. Applications of CFD to fermentation modeling include effects of stress on cell morphology and mass transfer from the bulk solution to the organisms [46]. Biochemical models should be coupled to the CFD models in order to give a closed link between biochemistry and fluid dynamics of the system [33]. Haringa assesses the effect of substrate heterogeneity on the metabolic response of *P. chrysogenum* in industrial bioreactors via coupling of a 9-pool metabolic model with Euler-Lagrange CFD simulations toward rational scale-down and design optimization [48].

Another way to construct mathematical models of microbial growth is the use of FFNs, which describe the behavior of different configurations of bioreactors. An example of this type of applications is the modeling of the production of bioethanol obtained from sugar beets [49]. Here, a three-layer FFN is used to describe the dynamic behavior of the reactor. The first neuron layer consists of system inputs, which correspond to substrate concentration, substrate type, and fermentation time. The second layer corresponds to hidden neurons that process the information through their activation function. Finally, the third layer matches the output of the system that corresponds to the viable cell count of yeasts and the concentration of ethanol produced. On the other hand, GBMs and their hybrid models are not only used to characterize fermentation kinetics but can also describe general behaviors of bioprocesses. For example, in fed batch cultures of *Chlorella pyrenoidosa*, a hybrid scheme of ANN with mass balance mechanistic models describes the general behavior of the states of the system, reducing considerably the variability of their predictions, and achieving versatility in application [50]. These types of GBMs are useful in cases of high complexity due to metabolic dynamics of microorganisms [51]. GBMs or hybrid models are not only combinations of first principles with ANNs, but there may also be hybrid models obtained through the combination of statistical models with ANNs. This type of models usually has special applicability in the optimization of operating conditions of bioreactors (e.g., fed batch fermentation of *Ralstonia eutropha* for poly-β-hydroxybutyrate production) [52].

Soft sensors are also useful in control design. For example, sliding mode observers can describe the behavior of sulfate reduction rate which results from *Desulfovibrio alaskensis* fermentation [53]. These observers use turbidimetric and

colorimetric titration information, and formulate based on sliding modes and sigmoidal functions, but their performance depends strongly on the nature of the system and its monitoring schemes.

Abrupt leaps in substrate concentration can be detected and prevented by the strategy of adaptive or optimal control by coupling with an observation scheme such as ANNs. For example, in L-glutamate production with *Corynebacterium glutamicum* fermentation, physical sensor applications are limited because of high costs and system complexity. However, it is possible to use simpler measurements such as oxygen concentrations, temperature, pH, and carbon dioxide production to train models of ANNs that can approximate the dynamic behavior of glucose concentration [54].

6. Conclusion

In bioprocesses, representation through simple or complex models, fermentation must consider process variables and analytes in mathematical models to achieve optimization, to develop simulations, and to calculate the output of critical variables in bioprocesses. Kinetic models allow predicting the behavior of biochemical reactions. This useful information is critical to techno-economic analysis. The incorporation of simple or complex models could represent phenomena more precisely and thus enhance our comprehension. In the design of bioreactors, a mathematical model is necessary to allow selecting the optimal operating conditions. There is a wide variety of types of models ranging from simple statistical descriptions to artificial intelligence tools. Appropriate model selection depends on the specific application: unstructured models can describe the global behavior, while unstructured models can describe specific phenomena such as metabolic pathways.

Another alternative in the modeling of bioreactors is the black or gray box models, which can be used for bioreactor design, without describing in detail the phenomenology present in the system, which is mainly focused on the global behavior of the system. An important part of the modeling, design, and control of bioreactors is the selection of appropriate sensors. It is often difficult to find suitable sensors for the process, so soft sensors are an interesting alternative to solve this problem.

Once a model describing the dynamical behavior of the bioreactor reaches the available condition, the control scheme can be designed. The goal may be different in each scenario: in the case of variables such as pH, this objective is usually regulation, but in variables such as concentrations and temperature, tracking is usually the goal. In any of these cases, slow and smooth dynamics inherent in these processes usually allow PID controllers to bring system states to the set point efficiently.

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Conflict of interest

The authors declare not to have a conflict of interest.

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Chapter 3

Small-Scale Process for the Production of Kefiran through Culture Optimization by Use of Central Composite Design from Whey and Kefir Granules

José Manuel Pais-Chanfrau, Lorena D. Carrera Acosta, Paola M. Alvarado Cóndor, Jimmy Núñez Pérez and Milton J. Cuaran Guerrero

Abstract

Cheese is one of the most demanded dairy products worldwide. However, during the conversion of milk to cheese, about 10 liters of milk are employed and about 9 liters of whey are generated for each 1 kg of cheese produced. The whey has traditionally been used for animal feed and as starting material for obtaining whey proteins. Furthermore, whey has the significant values of BOD and COD, becoming the most important contaminant in the dairy industry. For this reason, further growth of cheese sector is being limited by the surplus of whey as a by-product of the production of the cheeses. One of the many possibilities offered by the whey is its use as a starting material to produce many biotech products with a higher added value. The kefiran is a degradable biopolymer and is formed by galactose and glucose units, in almost similar proportions, which have been found with numerous benefits for human health. It is produced by a consortium of acid-lactic bacteria and yeasts, which coexist within the kefir granules, which are able to grow and multiply using the lactose present in the whey. The objective of the present study is to establish a small-scale process that allows the obtaining of kefiran.

Keywords: kefiran, central composite design, statistical optimization, *Lactobacillus kefiranofaciens*, kefiran production process

1. Introduction

Although it is still below the per capita consumption level recommended by FAO, milk production in Ecuador has been growing for some years now [1]. The stimulus of milk consumption and its derivatives was a state policy in recent years [2]. Within Ecuador, the Andean region marches at the head of production levels, accounting for about 77% of the country's production [3].

Of the 5.5 million liters of milk produced daily in Ecuador, 48% is sent to the industry, 35% of this goes to the industrial production of cheese [4], and about 12% of the total milk goes to the artisan cheese production. Therefore, around 47% of the total daily production of fresh milk is destined to produce different types of cheeses.

To produce 100 kg of cheese, about 1000 liters of fresh milk are used and about 900 liters of liquid milk whey (WL) are generated [5]. The WL is a yellowish liquid, whose characteristics vary with the region, the time of year, and the forage used in cattle feed. The majority composition is formed by 4.5–5% (m/v) of lactose, 0.6–0.8% (m/v) of whey proteins, 0.5% (m/v) fat, and 8–10% (m/v) (dry basis) mineral salts [6]. Its own composition gives the WL high values of COD and BOD, reporting values of 60–80 and 30–50 kg/m^3, for the COD and BOD_5, respectively [7, 8]. This by-product of the dairy industry, therefore, classifies as the most polluting effluent of this industry and constitutes the main impediment, for the further growth of the dairy industry [9].

There are numerous reviews on the use of whey as a raw material to obtain products with a higher added value than cheese or other dairy products [5, 10–12].

One of the products that can be obtained from WL by fermentation is the exopolysaccharides (EPS), which can be formed by sugars of equal or lower sweetening level than commercial sugar, but which can provide other beneficial effects for human health, classifying many of them as prebiotic substances [13].

Kefiran is an EPS, soluble in water, edible, and biodegradable, which is part of the structure of the kefir granule [14–17], which forms a symbiotic consortium of lactic acid bacteria (LAB), fungi, and yeasts, which can transform fresh milk in a fermented and effervescent milk drink, with a certain ethanol content, called kefir, very popular in central-eastern Europe and in Asia [18–21].

The role of kefiran seems to protect the microbiota inside the kefir granules [22]. Its synthesis is attributed to the LAB *Lactobacillus kefiranofaciens*, but it has been proven that its levels depend on the interactions that this bacterium establishes with others and the presence of some yeasts of the consortium present in the kefir granule [23, 24].

Kefiran, like kefir, has been attributed to several beneficial properties for human health, such as promotion of the growth of *Bifidobacterium bifidum* and modulating its genetic expression [25], its healing capacity [26, 27], hypotensive [28], hypercholesterolemia lowering [29, 30], anti-inflammatory agent [31], body weight regulator [32], immunoenhancer [33], and it has some anticancer effects [18, 34, 35]. It is for this reason that interest has recently grown to produce this EPS [24, 36, 37].

The production of kefiran goes through a fermentative phase where the polysaccharide is synthesized by *L. kefiranofaciens*, using a suitable carbon source such as lactose, present in the WL [38]. There are numerous reports suggesting that the temperature, the amount of inoculum, and the lactose concentration determine the amount and productivity of this phase [23, 24, 39]. However, the diversity of origins of the kefir granules and the logical variation of the amounts and types of microorganisms present in the granule mean that the optimal conditions may vary in each case and region.

2. Statistical optimization of kefiran production from whey and kefir granules by using response surface methodology

Response surface methodology (RSM) has been extensively used to find optimal fermentation conditions for the production of metabolites and chemicals from different microorganisms [40, 41].

Certain response (i.e., product concentration or productivity) depending for many well defines cultivation variables (like pH, temperature, substrate concentration, etc.) can be related via experimentation through a mathematical model with certain response like concentration or productivity of a metabolite produce by microorganism. Such a model can be used, after its verification, to "navigate" inside defined region of variables, to find the combination of such a variable able to maximize or minimize the response function [42–45].

Some designs of experimental (DOE) arrangements have been employed in RSM. One of the most popular is the central composite design (CCD), which the experimental points surrounded certain central point in equidistance from them [46].

In CCD, the extreme values of the response are searching inside a square in scripted in the sphere formed by the experimental points. In the CCD, in its minimal expression, only the central point needs to be replicated, assumed that the statistical variation of the rest of all experimental points is the same that in the central one. Additional point, however, can enhance the accuracy of the model. With this kind of DOE, a quadratic model of response could be obtained. A second degree of a polynomial can be used to find the extreme values (minimum or maximum) inside the experimental surface. Before that, however, it can be demonstrated that the quadratic model of response is suitable to navigate inside his values to find the optima. Analysis of variance (ANOVA) of the mathematical model and the analysis of his residuals can be served for this purpose.

Several professional statistical software can be used to perform the planning and analysis of the mathematical model. Design-Expert 11, from the Stat-Easy, Inn. (Minneapolis, USA) was used through this work.

In previous work, whey powder (WP) and kefir grains have been used to find the suitable conditions for the WP concentration and temperature to maximize kefiran production [37] or maximize certain desirability function formed by equally weighed kefiran as a prebiotic, and the cell concentration of LAB and yeast in a functional drink [47].

In those works, however, some inconveniences associated to cost of WP as a starting raw material has been detected when the preliminarily economic analysis was performed, suggesting the direct use of a cheaper sweet or acidic whey instead of the WP for similar purposes.

The way of optimization passes for a well-designed experiment where the response (concentration of kefiran in the fermentation broth) is correlated with relevant independent variables, like concentration of liquid sweet whey (WL) in the culture medium and temperature. The optimization algorithm used is shown in **Figure 1**.

For its simplicity, a central composite design was selected, bringing as a central point 25°C and 42% (m/m), for temperature and WL, respectively. A temperature of 25°C brings a maximum value of kefiran concentration in previous work with the same origin kefir grains ("Yogurt-Kombucha-Tibicos en Ecuador," Quito, Pichincha, Ecuador, www.kefir.ec), but employing whey powder as a lactose source [37].

Kefiran was determined according to phenol-sulfuric acid method [48] which employed the glucose as a reference sugar similar as it had been determined elsewhere [24, 37].

Due to a complexity of the consortia of microorganism presented in the kefir grain, a culture medium is supplemented either with glucose or sucrose, adjusted the brix of the medium at a constant value of 14%, as was suggested elsewhere [43, 44], and it was performed in previous work [37].

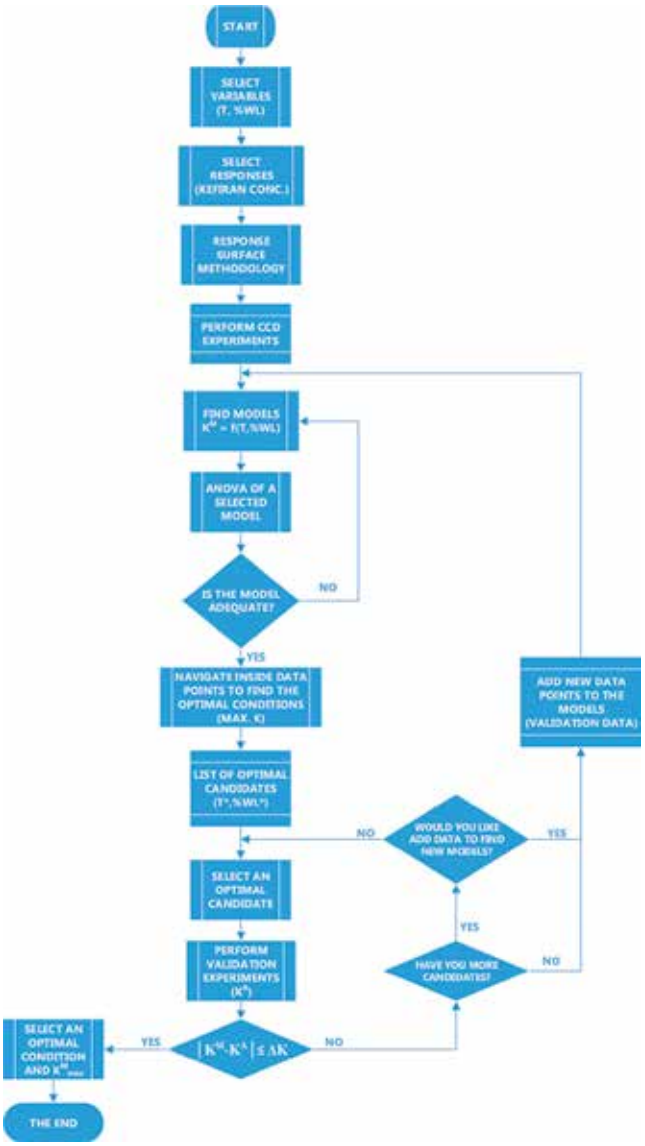

Figure 1.
Flow-chart to find the maximum of kefiran production by using CCD in RSM.

The starting CCD experiments for two independent variables (A: Temp and B: %WL) at a minimum of 13 points experiments are recommended [46], five of which corresponds to the central point and the rest of eight surrounding the central point. Due to the existence of position in a shaker, however, two experiments per point surrounding four experiments for a central experiments points were performed (**Figure 2**).

Table 1 shows the coded and actual values of the independent variables and the corresponding value for the response variable.

As a response range was a ratio of max to min of 12.4082, the response could be transformed. In this case, a power transformation was selected, which brings a model for coded variables of:

$$(Kefiran + 10.0)^{0.28} = 4.33 + 0.2231\,A + 0.0665\,B + 0.2322\,AB - 0.2233\,A^2 - 0.2107\,B^2 + 0.3001\,A^2B - 0.7354\,AB^2 - 0.2848\,A^2B^2 \quad (1)$$

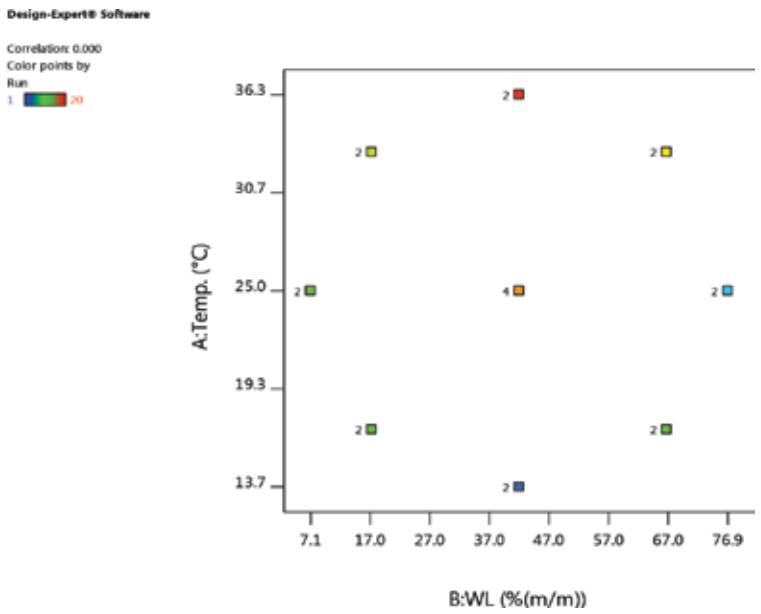

Figure 2.
Original surface data for independent variables.

Std	Code variables		Actual variables		Response
	A: Temp.	B: WL	A: T, °C	B: WL, % (m/m)	Kefiran, g Glu/ml
1	−1.000	−1.000	17.0	17.3	130.0
2	1.000	−1.000	33.0	17.3	17.8
3	−1.000	1.000	17.0	66.7	171.0
4	1.000	1.000	33.0	66.7	105.5
5	0.000	−1.414	25.0	7.1	109.0
6	0.000	1.414	25.0	76.9	129.7
7	0.000	0.000	25.0	42.0	180.7
8	0.000	0.000	25.0	42.0	182.4
9	0.000	0.000	25.0	42.0	173.3
10	0.000	0.000	25.0	42.0	171.4
11	−1.414	0.000	13.7	42.0	84.7
12	−1.414	0.000	13.7	42.0	82.4
13	−1.000	1.000	17.0	66.7	161.1
14	−1.000	−1.000	17.0	17.3	128.8
15	0.000	1.414	25.0	76.9	132.5
16	0.000	−1.414	25.0	7.1	108.6
17	1.000	1.000	33.0	66.7	87.6
18	1.000	−1.000	33.0	17.3	14.7
19	1.414	0.000	36.3	42.0	169.7
20	1.414	0.000	36.3	42.0	145.8

Table 1.
Coded and actual values of independent variables and its corresponding values of response.

ANOVA for reduced quadratic model for the transformed response of kefiran is shown in **Table 2**.

The model *F-value* of 190.66 implies that the model is significant. There is only a 0.01% chance that an F-value that is large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2, A^2B, AB^2, A^2B^2 are significant model terms.

The accuracy of the model can be judged by the plot of the actual responses *versus* predicted responses (**Figure 3**), and the normal plot of residuals (**Figure 4**).

The *predicted* R^2 of 0.9729 is in reasonable agreement with the *adjusted* R^2 of 0.9876; i.e., the difference is less than 0.2.

Adeq. precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 45.727 indicates an adequate signal. This model can be used to navigate the design space.

The *fit statistics* of the model is:

Std. dev.	**0.0597**	**R^2**	**0.9928**
Mean	3.86	Adjusted R^2	0.9876
C.V. %	1.54	Predicted R^2	0.9729
		Adeq. precision	45.7268

After checking the model for kefiran, an optimization procedure to find the maximum of kefiran was performed. A wide range of region for temperature and WL concentration was found, and suggested a combination of 90 points of temperature and WL concentration for the maximum of kefiran concentration.

Taking one of this point is to perform the validation experiments and confirm or refuse the model for the kefiran. This point corresponds with a combination of temperature and WL concentration of 30.5°C and 47.8% (m/m), respectively (**Figure 5**).

Three experiments at the selected condition were performed, reaching values of kefiran concentration of 181.1, 146.6, and 167.9 g Glu/ml. As can be seen, only the first are located inside the range suggested by the model, whilst two are outside. These results cannot confirm the validity of the model of kefiran.

Source	**Sum of squares**	**df**	**Mean square**	**F-value**	**P-value**	
Model	5.43	8	0.6786	190.66	<0.0001	Significant
A-T	0.3981	1	0.3981	111.86	<0.0001	
B-WL	0.0353	1	0.0353	9.93	0.0092	
AB	0.4312	1	0.4312	121.16	<0.0001	
A^2	0.3990	1	0.3990	112.11	<0.0001	
B^2	0.3553	1	0.3553	99.84	<0.0001	
A^2B	0.3602	1	0.3602	101.22	<0.0001	
AB^2	2.16	1	2.16	607.79	<0.0001	
A^2B^2	0.3245	1	0.3245	91.19	<0.0001	
Pure error	0.0391	11	0.0036			
Cor total	5.47	19				

Table 2.
ANOVA for the kefiran model.

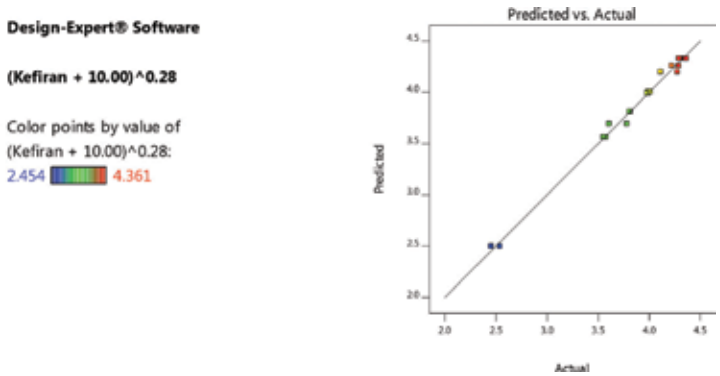

Figure 3.
Actual versus *model responses of the model for kefiran.*

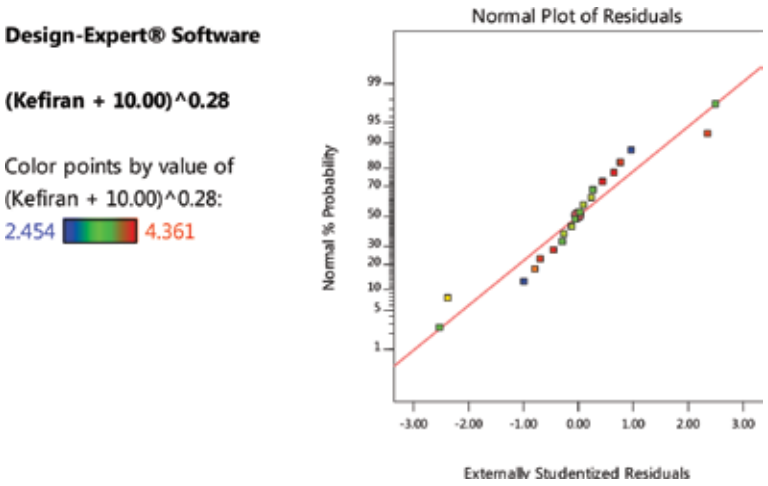

Figure 4.
Normal plot of residual in the kefiran model.

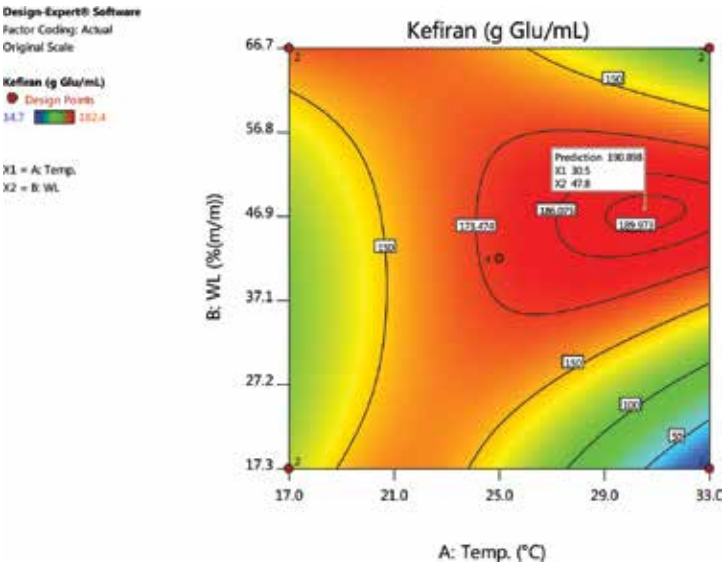

Figure 5.
Contour plot of the point selected for the validation experiments.

To check the usefullness of the kefiran model (Eq.1), one of the suggest combination of temperature (30.5°C) and sweet liquid whey (47.8% (m/m)) that should maximize the kefiran concentration was used to perform three validation experiments. Those experiments could not validate the model (**Table 3**).

According to the flow-chart shown in **Figure 1**, at this point could be evaluated another of 90 data points suggest in the optimization procedure to maximize kefiran for this model and perform with him the additional validation experiments or, alternatively, can be add the values of validation experiments to the originally data and find a new, more robust, suitable cuadratic model for determination of kefiran. The second alternative was followed, and a new set of experimental points was obtained (**Figure 6**).

The coded and actual quadratic model for kefiran were, respectively:

$$\begin{aligned} Kefiran = {} & 176.75 - 8.56\,A + 7.88\,B + 10.91\,AB - 28.15\,A^2 \\ & -28.50\,B^2 + 21.35\,A^2B - 54.50\,AB^2 + 17.40A^3 - 18.24\,A^2B^2 \end{aligned} \tag{2}$$

$$\begin{aligned} Kefiran = {} & -1017.04387 + 132.1778\,T + 12.3776\,WL - 1.6439\,T{\bullet}WL \\ & - 4.3795\,T^2 - 0.059484\,WL^2 + 0.052743\,T^2{\bullet}WL \\ & + 0.012188\,T{\bullet}WL^2 + 0.033978\,T^3 - 0.000467\,T^2{\bullet}WL^2 \end{aligned} \tag{3}$$

ANOVA, the model for kefiran concentration (**Table 4**), its fit statistics (**Table 5**), and the plot of predicted *vs* actual kefiran concentration values (**Figure 7**) suggests that the model could be used to find inside the space of independent variables the maximum value of kefiran.

Response	Predicted mean	Predicted median*	Std. dev.	n	95% PI low	Data mean†	95% PI high
Kefiran	190.9	190.7	9.7	3	175.5	**164.8**	206.9

**For transformed responses, the predicted mean and median may differ on the original scale.*
†For transformed responses, the data mean is calculated on the transformed scale.

Table 3.
Confirmation experiments for the first kefiran model.

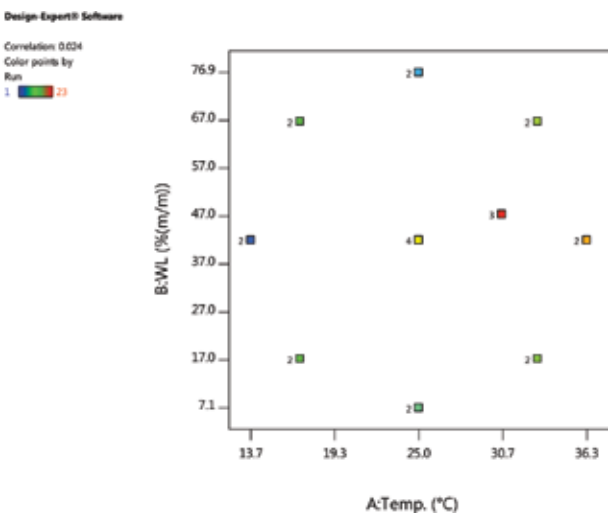

Figure 6.
New design space data of independent variables.

The *model F-value* of 60.14 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. *P-values* less than 0.0500 indicate model terms are significant. In this case, B, AB, A^2, B^2, A^2B, AB^2, A^3, A^2B^2 are significant model terms. The model term A is insignificant, but it counts to support hierarchy of the model (**Table 4**).

The fit statistics of the quadratic model for the kefiran concentration is shown in **Table 5**. The *Predicted* R^2 of 0.9307 is in reasonable agreement with the *Adjusted* R^2

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	50004.24	9	5556.03	60.14	< 0.0001	significant
A-T	39.19	1	39.19	0.4242	0.5262	
B-WL	497.29	1	497.29	5.38	0.0372	
AB	952.66	1	952.66	10.31	0.0068	
A^2	6339.38	1	6339.38	68.62	< 0.0001	
B^2	6498.00	1	6498.00	70.34	< 0.0001	
A^2B	1823.85	1	1823.85	19.74	0.0007	
AB^2	4845.51	1	4845.51	52.45	< 0.0001	
A^3	584.72	1	584.72	6.33	0.0258	
A^2B^2	1330.43	1	1330.43	14.40	0.0022	
Pure error	1200.93	13	92.38			
Cor total	51205.17	22				

Table 4.
ANOVA of the quadratic model for kefiran concentration.

Std. dev.	**9.61**	**R^2**	**0.9765**
Mean	129.67	Adjusted R^2	0.9603
C.V. %	7.41	Predicted R^2	0.9307
		Adeq. precision	25.3566

Table 5.
Fit statistics of the quadratic model for kefiran concentration.

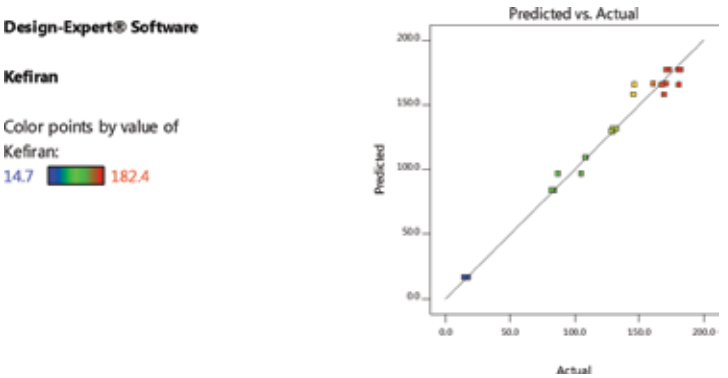

Figure 7.
Predicted versus *actual values of kefiran concentration.*

of 0.9603; i.e., the difference is less than 0.2 *and Adequate precision* measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 25.357 indicates an adequate signal. This model can be used to navigate the design space.

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A:T	Is in range	17	33	1	1	3
B:WL	Is in range	17.3	66.7	1	1	3
Kefiran	Maximize	14.7	182.4	1	1	5

Table 6.
Constrains to find the maximum of kefiran concentration.

Number	T, °C	WL, %(m/m)	Kefiran, mg Glu/ml	Desirability
1	23.864	45.932	178.090	0.974
2	19.962	66.700	174.901	0.955

Table 7.
Solution candidates to values for independent variables to reach maximum for the kefiran concentration.

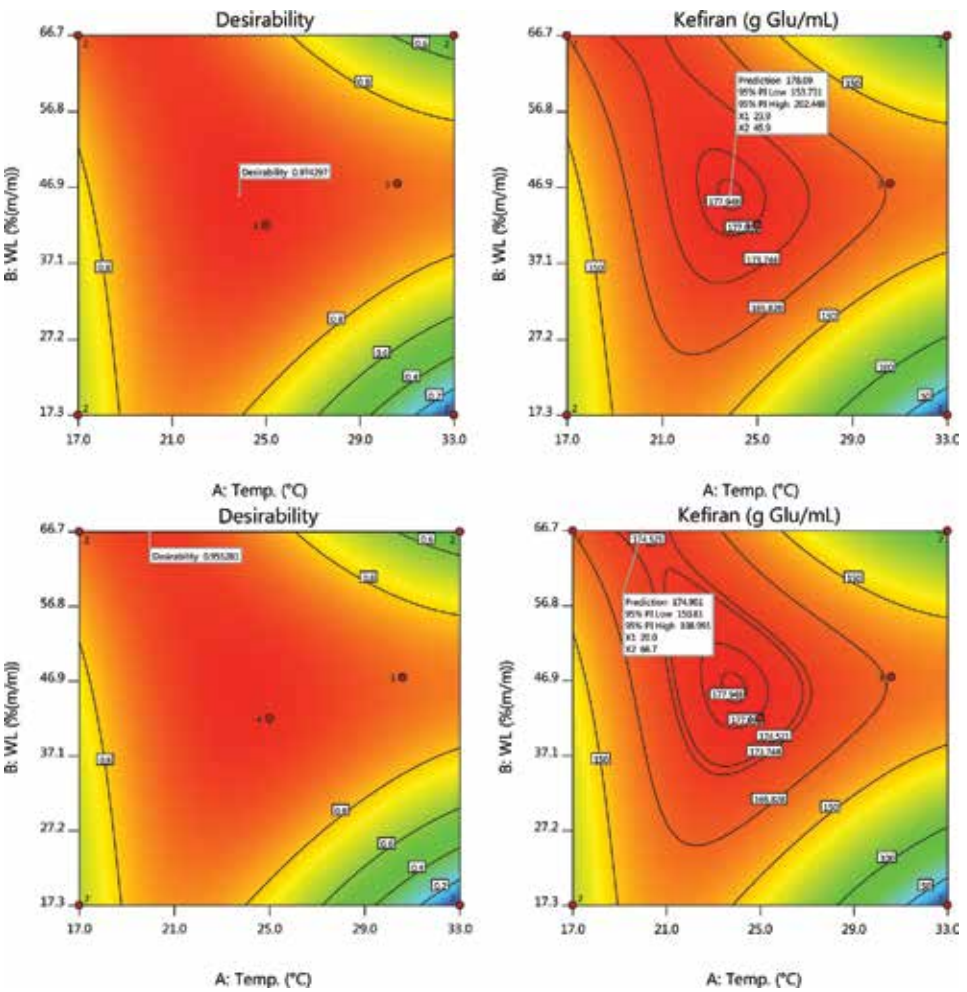

Figure 8.
Contour plots of solution values for independent variables and candidates to reach a maximum for kefiran concentration.

Response	Predicted mean	Std. dev.	n	SE Pred	95% PI low	Data mean	95% PI high
Kefiran	178.1	9.6	3	8.1	160.7	177.5	195.5

Table 8.
Value of the validation experiments for two-side confidence of 95%.

A numerical optimization to find the maximum of kefiran concentration inside the space of independent variables with constraints shown in **Table 6** was performed.

And two, equally in possibilities, solution candidates to reach a maximum for the kefiran concentration were obtained (**Table 7**).

The first suggested solution (23.9°C and 45.9% (m/m) for temperature and whey liquid, respectively) was selected to perform the three validation experiments.

The validation experiments for the kefiran concentration were 175.5, 184.5, and 172.6 mg Glu/ml, reaching a mean of 177.5 ± 6.2 mg Glu/ml, which is inside the probability interval of the model. In this manner, the validation experiments confirm the adequateness of the kefiran model (Table 8).

Further experiments to establish the small-scale production of kefiran were conducted starting with a fermentation stage with the same media, inoculum concentration, and a temperature and concentration of whey at the optimal conditions find here.

3. Small-scale production of kefiran

The soluble exopolysaccharide kefiran, synthetized by *Lactobacillus kefiranofaciens* as a part of kefir granules, could be isolated and purified from the culture broth after the lactose-fermentation from whey [37, 49]. To achieve this, several isolated steps can be executed like filtration, homogenization, thermal inactivation of glycolytic enzymes, polysaccharide precipitation, separation, and washing the precipitate (**Figure 9**).

Due to kefiran representing 50% (dry basis) of a granule [50], after fermentation, the broth could be homogenized to use all the granules to isolate the kefiran. However, together with kefiran, certain contaminants and glycolytic enzymes that eventually could degrade the kefiran itself will be aggregated. For this reason, it could be convenient to ask the question if it is necessary to employ the disrupted whole fermentation broth including in it the kefir granules to isolate kefiran, or only use the supernatant of the culture after separate the kefir granules from the broth of culture.

Another question to ask is, if the kefiran grains is separated from the supernatant is the option choice, how many times the kefiran granules can be reused? Will it be convenient to reuse many times and replace the inoculum at all or to refresh inoculum partially with a fresh grain?

At this point, at least until three times was reused, the same kefiran granules with the washing the granules with abundant sterilized distillate water between batches, with no significant difference in the final kefiran concentration (results not shown). Further experiments, however, should be performed to know how many times this process could be repeated.

Either two alternatives can be implemented instead, but only if the homogenization of whole fermentation broth brings a significant increment in the kefiran concentration in respect to use the supernatant. Additionally, in the case that only the culture supernatant was used, less time and energy would be used, and the kefir

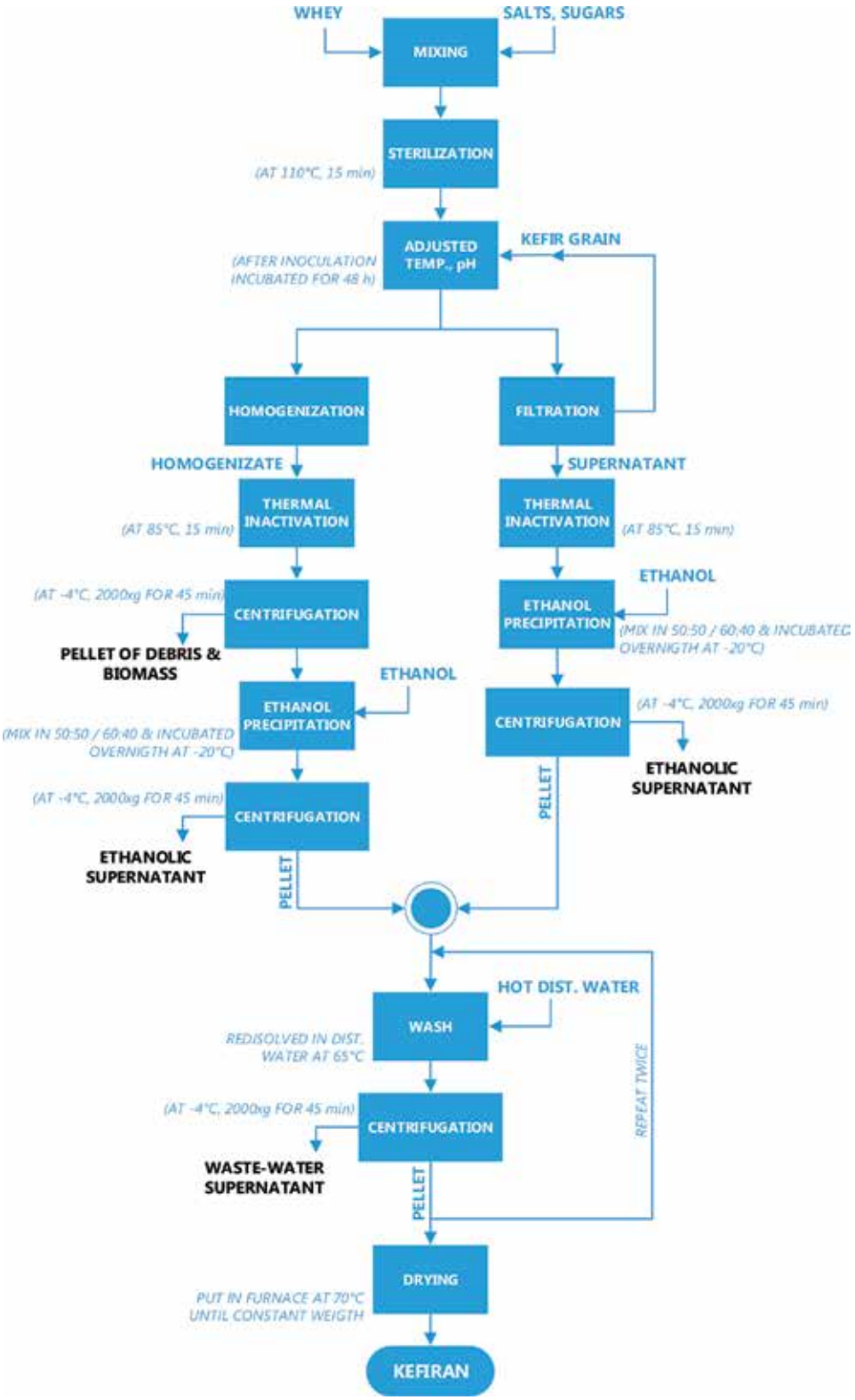

Figure 9.
Lab-scale procedure for the kefiran isolation from the fermentation of whey with kefir granules.

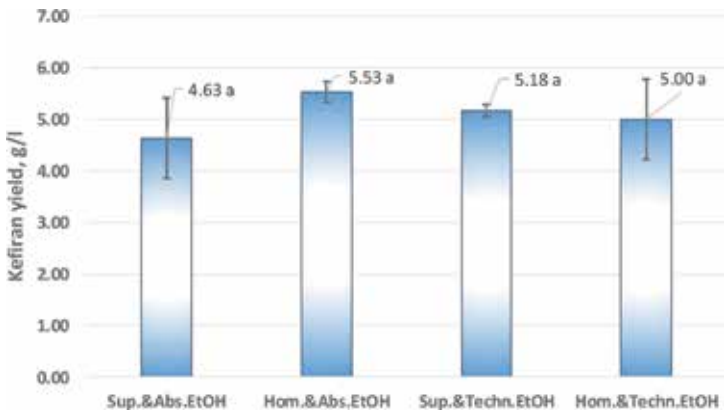

Figure 10.
Experiments to compare supernatant versus *homogenate, and ethanol absolute (anhydrous)* versus *technical in the yield of kefiran. Legend: Sup—supernatant, Hom–homogenate, Abs—EtOH-absolute ethanol, and Techn. EtOH—technical ethanol (96% (v/v)).*

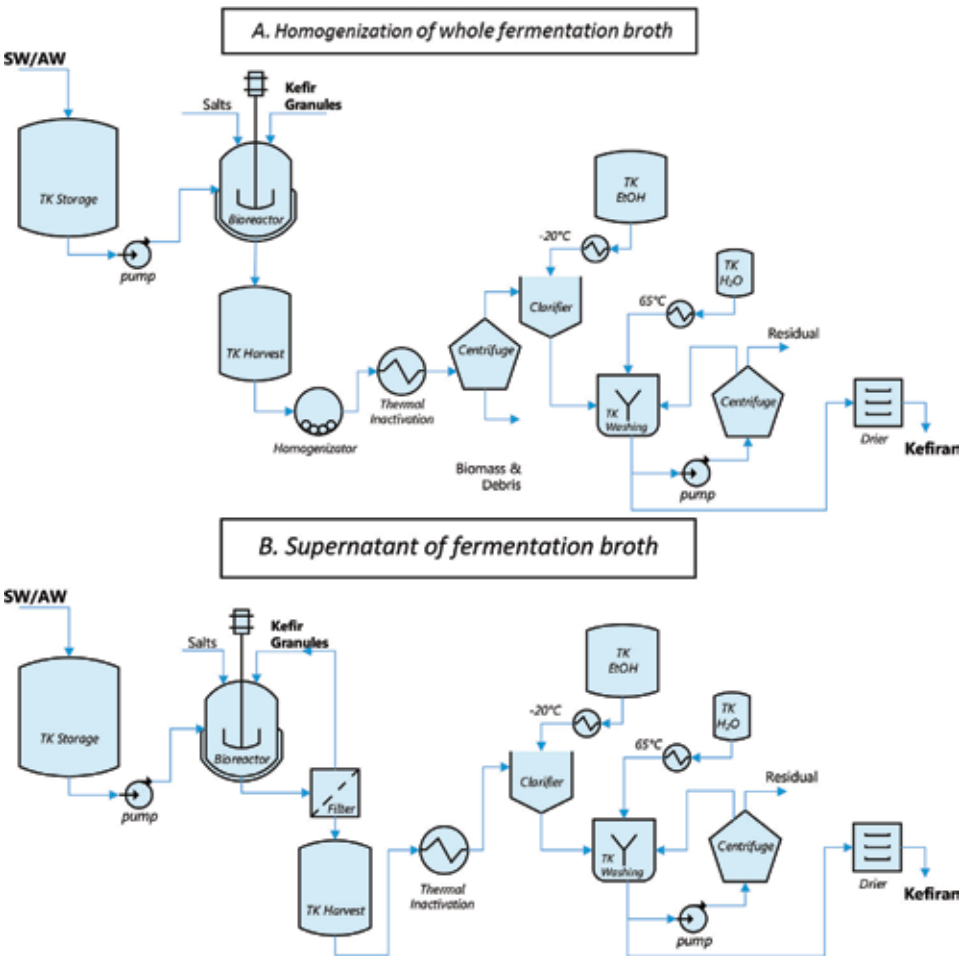

Figure 11.
Two possible flow diagrams for the kefiran production, by using: (A) the homogenization of whole fermentation broth and (B) the supernatant of fermentation broth. Legend: SW/AW: sweet or acidic whey, EtOH: ethanol, and TK: tank.

grains can be reused inside the process and wash residuals should be less contaminated that the similar is obtained for the homogenization variant.

Another question is associated to the use of solvent precipitation. Several reports referred the use of absolute (anhydrous) ethanol [24, 38], with a higher price respect his counterpart, the technical ethyl alcohol at 96% (v/v). If the technical ethyl alcohol at 96% (v/v) could be employed instead the more expensive absolute (anhydrous) one during the solvent precipitation of kefiran, a significant impact over the manufacturing cost of kefiran could be obtained.

At this point, three independent experiments were conducted to compare homogenization *versus* supernatant alternatives, and the utilization of absolute (anhydrous) *versus* technical ethyl alcohol as the precipitation agent. When the precipitation experiments were performed using absolute alcohol (anhydrous) a proportion of 50:50 with a material was employed; and when technical alcohol at 96% was used instead the anhydrous ethanol, the technical alcohol: material was mixed in a proportion of 60:40. Each experiment was started with the same fresh whey and inoculum and was performed as shown in **Figure 9**.

The alternatives shown in **Figure 10** do not have significant differences ($p > 95\%$) between them, suggesting that the more suitable and economic way for production of kefiran is to use the supernatant of fermenting broth and technical ethanol as a precipitation solvent (**Figure 11B**), instead of the homogenization of whole broth and using absolute ethanol as a solvent (**Figure 11A**).

Kefiran isolation process was carried out from three 100 ml batches each, yielding 5.2 ± 0.1 g/l of kefiran in the culture supernatant.

These results are slightly higher than the 3.1 ± 1.3 [37] or 1.91 g/l of kefiran [24] reported recently, and higher to the values between 1.5–3.7 g/l reported previously [38, 51].

4. Conclusions

The usefulness of the RSM was demonstrated in the search for a condition where the concentration of kefiran is maximized from liquid sweet whey, significantly cheaper than whey powder. It was also possible to satisfactorily replace the "absolute" (anhydrous) ethyl alcohol with the technical-grade ethyl alcohol at 96%(v/v). Both facts represent a remarkable financial saving in the production of the kefiran, which would allow to begin the studies to scale-up the production technology of this attractive EPS.

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Chapter 4

Catalyst Development of Microbial Fuel Cells for Renewable-Energy Production

Masayuki Azuma and Yoshihiro Ojima

Abstract

In this chapter, we focus on microbial fuel cells (MFCs) that convert the energy from organic matters into electrical energy using microorganisms. MFCs are greatly expected to be used as a relatively low-cost and safe device for generating renewable energy using waste biomass as a raw material. At present, however, it has not reached the desired practical application because of the low-power generation; hence, improvements on fuel cell efficiency, such as electrode materials, are still being examined. Here, we focus on the microorganisms that can be used as catalysts and play a central role in improving the efficiency of the fuel cells. Several kinds of microbial catalysts are used in MFCs. For example, *Shewanella oneidensis* has been well studied, and as known, since *S. oneidensis* transports the electrons generated within the cell to the surface layer, it does not require a mediator to pass the electrons from the cells to the electrode. Furthermore, *Escherichia coli* and *Saccharomyces cerevisiae*, a model organism for MFCs, are also used. The improvements of such microbial catalysts have also been proceeding actively. Here, we elaborated on the principle of MFCs as well as the current situation and latest research on the catalyst development.

Keywords: microbial fuel cell, MFC, catalyst, renewable energy, bioelectrochemical device, microorganism, biomass

1. Introduction

The fossil fuel depletion and inevitable global warming have become worldwide problems; thus, significant efforts have been made to generate and utilize renewable energy to alleviate these crises. Methods for obtaining energy compounds from biomass, such as ethanol, methane, and hydrogen, have been developed using environment-friendly technology, and some of these technologies have been put to practical use. It is important to establish the technologies that are able to obtain energy in various forms according to the environments and circumstances of each region. Apart from the above technologies, biofuel cells utilizing microorganisms and enzymes, which can generate renewable electrical energy from organic matters contained in biomass, begin to attract attention as a means to obtain sustainable energy. It has not been put into practical use yet, but without the problem of by-products, electricity can be directly obtained from the devices, whereby multiple operations, such as product distillation (e.g., ethanol), are not necessary. Moreover,

if biomass waste is used as the fuel, no food competition will occur. Therefore, using this method, energy can be obtained sustainably (**Figure 1**).

There are various types of biomass, e.g., sustainably harvested wood, waste paper, food waste, sewage sludge, and various wastewaters. Taking wood-based biomass as a fuel example, when everything is burnt using available technology for thermal power generation, there will be nothing left, and we will lose some other useful compounds contained in it. On the other hand, in biofuel cells, although electricity is generated from the sugar obtained from the biomass, other components in the wood, such as lignin, can be used for purposes other than power generation. Generally, the energy density of the biomass used as a fuel for MFCs is high. For example, glucose and xylose, found in various plant biomass, can produce up to 20 or 24 electrons per molecule, provided that they are completely oxidized to carbon dioxide. It is possible to generate 4430 Wh power per kg of glucose according to the calculation described later. For reference, a typical lithium-ion battery has a weight energy density of about 200 Wh per kg. This comparison means that glucose and xylose are two biofuel sources of interest, especially as electron donors. Therefore, MFCs using glucose and/or xylose as their fuel have great potential as a means of obtaining high energy.

In biofuel cells, biological reactions are used for the oxidation reaction of biomass, and they are divided into two based on the type of catalyst used: (1) enzymes and (2) microorganisms. When enzymes are used, the most widely studied mechanism is the two-electron oxidation system by glucose oxidase (GOx) or glucose dehydrogenase (GDH) [1]. Since purified enzymes are generally used, the reaction rate is faster than using microorganisms. However, the number of electrons obtained by one enzymatic reaction is smaller than that of a microorganism. For example, when GDH is used as the catalyst, glucose is oxidized to gluconic acid, and at most, only two electrons are obtained from one glucose molecule. Therefore, if only one enzyme is used, high-energy production per glucose consumed cannot be much expected. Further, the addition of cofactors, such as nicotinamide adenine dinucleotide and pyrroloquinoline quinone (PQQ), is necessary for the enzymatic reaction to enhance the energy production. Furthermore, the cost of enzyme purification is also high; hence, enzymes are better utilized in sensor applications than energy production. By contrast, as mentioned above, one completely oxidized glucose molecule gives 24 electrons when using microorganisms as the catalyst. It

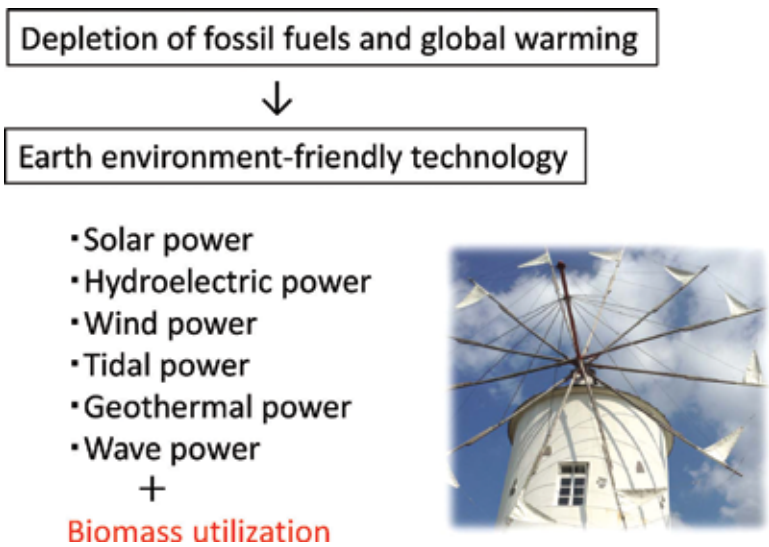

Figure 1.
Environmentally friendly energy.

shows a possibility of obtaining more electrons per glucose consumed. Moreover, the addition of cofactors is not necessary, unlike when enzymes are used. These are some substantial advantages of MFCs. However, the production of low power in MFCs is still a problem because of typical processes in living organisms, such as the uptake of glucose into cells, metabolism repression, and extraction of electrons from the inside of cells (**Figure 2**). Many researchers are working to solve such problems, and those results are summarized in recent review articles [2, 3].

Looking back at the historical background, research on the MFCs has been conducted for a long time, whereby the first idea of using microorganisms to produce electricity was conceived and reported by Potter in 1911 [4]. *Escherichia coli* and *Saccharomyces cerevisiae* were used as the catalyst, and platinum was used as the electrode. Further, in 1931, Cohen showed that a number of small fuel cells connected in series produced 2 mA of electricity at over 35 V [5]. Early MFCs used an artificial mediator, e.g., thionine, methyl viologen, and humic acid, to carry electrons from the microbial cells to the electrodes [6, 7]. The oxidized mediators came into contact with the microbial cells, and were reduced by accepting electrons, and they were then separated from the microbial cells. They diffused and came into contact with the surface of the electrode to release the electrons and were reoxidized thereafter. Overall, the addition of artificial mediators promotes the flow of electrons. Next, in the 1990s, several bacteria were found capable of acquiring electrons from the electrode via a self-mediator without the addition of an artificial mediator. Moreover, they used electrons for their growth; for example, a ferric-iron-reducing bacterium *Shewanella putrefaciens* grew on lactate by obtaining electrons from the electrode [8], and similar reports were found on *Shewanella oneidensis* and *Geobacter sulfurreducens* [9, 10]. Although the flow direction of electrons between cells and electrodes is opposite from the MFC explanation just before, these findings led to the development of mediator-free MFCs [11–13]. In the 2000s, the mechanism at the cell surface whereby bacteria directly came into contact with the electrodes and carried the electrons was reported [14, 15]. Since then, MFC research, including the analysis of the electron transport mechanism at the cell surface, has been actively conducted. In fact, the number of publications related to the MFCs grew significantly since 2010 and reached nearly 1000 in 2016 and 2017 according to ISI WEB OF SCIENCE [2]. In particular, with the discovery of new fuel cell components other than the microbial catalysts, the performance of MFCs could be increased further.

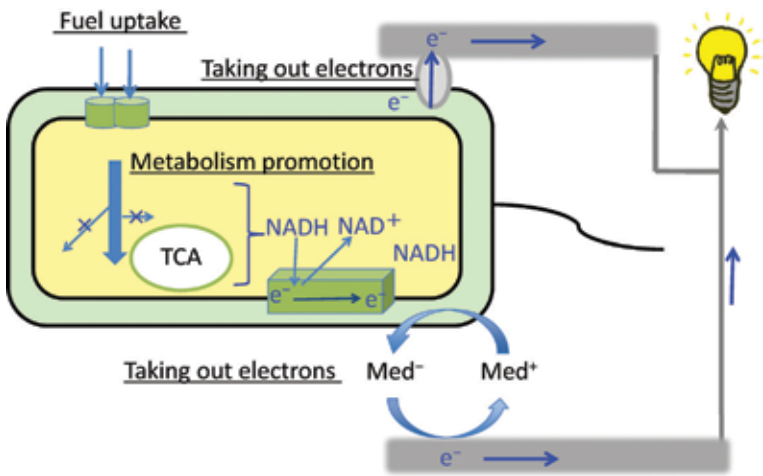

Figure 2.
Electron generation and extraction in the microbial fuel cell system. Med: mediator.

The performance of MFCs is evaluated based on some indicators. The electrical energy (Wh) used to express the capacity of dry batteries is also an important indicator, but only a few papers have reported it so far. In most cases, the performance is expressed as the maximum power per anode electrode area (power density per area) or the maximum power per cell volume (power density per volume). The latter is a straightforward index and important for practical use. For example, a relatively high-performance small-scale fuel cell (2.5 mL) using a complex (mixed) microorganism system was reported in 2007 with a power density of 1550 W/m^3 [16]. Other fuel cells performing beyond 500 W/m^3 were also reported [17–22], but many of them are still at a microliter or milliliter scale. Owing to the low proton diffusion speed and high internal resistance, the maximum power per volume tends to be small for a large-scale fuel cell. MFCs with a volume more than 1 L were also being studied in the laboratory, but the maximum power per volume was still low at the level of several W/m^3 to tens of W/m^3 [23, 24]. Scaling-up is also another issue of MFCs, and further improvements are still being conducted.

Practical applications of MFCs are still problematic because of the high cost and low-power generation. Despite this situation, research on the implementation of MFCs has been carried out. For example, an artificial stomach called Gastrobot (aka Chew-Chew train) using *E. coli* and sugar as the catalyst and fuel, respectively, EcoBot-III (a self-sustainable robot with its own circulatory system, such as ingestion, digestion, and excretion), and several environmental sensors using the MFCs for powering [2]. Such implementation studies are also important to understand the desired performance level for MFCs. Meanwhile, in order to bring MFCs closer to practical use, a combination of power generation and other effects is one of the promising methods. For example, MFCs that are installed at a wastewater treatment facility are expected not only to reduce the generated sludge amount from the treatment but also to cover a part of the electricity load used by the plant. Recently, the minimum performance of MFCs required for reaching energy neutrality in a wastewater treatment facility has been calculated [25], and the realization has been highly expected. Such research on MFCs installed for wastewater treatment has been actively carried out so far, and the experiments were examined at a pilot-scale plant of more than 10 L [26–28]. Accordingly, the practical use of MFCs is expected soon. Besides this, although it deviates from energy production, the use of MFCs as a sensor has also been extensively studied. In order to perform on-site real-time monitoring, it is important to recognize the toxic compounds rapidly. Several analytical techniques based on electrochemistry have been developed for this purpose, but in many cases, they lack practicality for environmental measurements. The MFC-based biosensor is one promising candidate, and it has already been shown that not only toxic heavy metals but also toxic organic compounds can be detected [29]. The sensitivity adjustment suitable for the detection of specific contaminants is left as a challenge, but there is still a great expectation for its application.

Here, we will explain the mechanism of electron generation in microorganisms, introduce the principle of MFCs, describe the microbial catalysts used for various MFCs mentioned above, and discuss the recent topics on microbial catalysts.

2. General principles of MFCs and various microbial catalysts

2.1 Mechanism of electron generation in microbial cells

MFCs utilize the decomposition energy of organic matters by the organisms to produce ATP, known as the energy currency, based on the energy obtained from

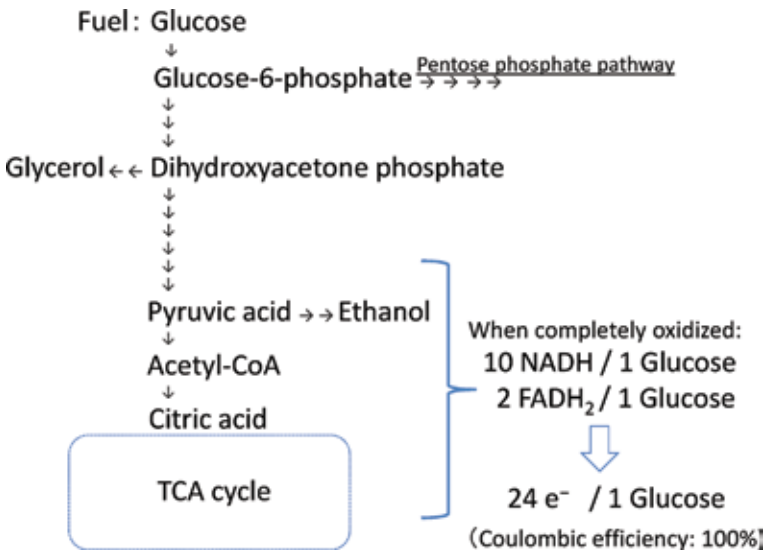

Figure 3.
Glucose metabolism (Saccharomyces cerevisiae).

this process. Taking glucose decomposition in *Saccharomyces cerevisiae* as an example (**Figure 3**), glucose taken into the microbial cells by cell membrane enzymes is oxidized and decomposed to pyruvic acid by various enzymes via an intracellular glycolysis system. Next, pyruvic acid becomes carbon dioxide and water when it is completely oxidized via the TCA cycle. The electrons generated are then collected in the mitochondrial inner membrane in eukaryotes, and in prokaryotes, they were accumulated in the cell membrane via NADH and FADH2 (both of which provide two electrons to the membrane electron flow; **Figure 3**). In addition, the flow of electrons in these membranes is maintained through multiple protein complexes localized in the membrane. Quinone compounds and cytochrome proteins are also included in the complex. ATP is synthesized by the membrane enzyme, ATP synthase, using the proton concentration gradient, which is generated inside and outside the membrane because of the flow of electrons. In MFCs, it is thought that it takes the electrons directly from NADH or the flow of electrons generated in the membrane by the decomposition of organic substances. A part of the electrons generated within the microbial cell is carried to an electrode outside the cell via an electron carrier called a mediator. When one molecule of glucose is completely oxidized into carbon dioxide and water in the cell, 10 molecules of NADH and 2 molecules of FADH2 are also generated. In total, 24 electrons are obtained from 1 glucose molecule. If this principle were to apply to fuel cells, the Coulomb efficiency, which is an index of energy efficiency, would become 100%. Therefore, in order to obtain electrons more efficiently from the cells via such metabolism, it is important to adjust the redox balance within the microbial cells in the MFCs.

2.2 Calculation of the energy obtained from glucose

In the case of using glucose as the fuel source, the reaction occurring in the anode tank is represented by Eq. (1), and the reduction reaction occurring in the cathode tank is represented by Eq. (2).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad (1)$$

$$6O_2 + 24\,H^+ + 24\,e^- \rightarrow 12\,H_2O. \quad (2)$$

The oxidation-reduction potential of Eq. (1) is −0.42 V, whereas the oxidation-reduction potential of Eq. (2) is 0.82 V. Therefore, the total potential difference of the MFC reaction (Eq. (1) + Eq. (2)) as represented by Eq. (3) is 1.24 V. Theoretically, the voltage exceeds 1 V, but in most cases, it has never reached that value.

$$C_6H_{12}O_6 + 6O_2 + 6H_2O \rightarrow 6CO_2 + 12\,H_2O. \quad (3)$$

Assuming that 24 electrons are obtained from 1 glucose molecule and that they can be recovered in 1 h, the quantity of electricity (Ah) obtained from the glucose (1 kg) can be calculated using the Faraday constant (96,485 C/mol) as shown in Eq. 4. As a result, the electrical energy of 4430 Wh can be achieved if the potential is 1.24 V; accordingly, this value is the same as the value mentioned in the Introduction section.

$$(24 \times 96{,}485 \times 1000)/(3600 \times 180) = 3574\ \text{Ah}. \quad (4)$$

2.3 Basic components of dual-chambered MFCs using a mediator

A dual-chambered fuel cell consisting of an anode tank and a cathode tank is the simplest and has been used for a long time for MFCs. In many cases, they are separated by a cation exchange membrane (CEM) to create a potential difference between the two tanks (**Figure 4**). CEM prevents mixing of each content and allows the protons generated in the anode to migrate to the cathode. In addition, CEM selection, especially based on its proton transfer efficiency, is important

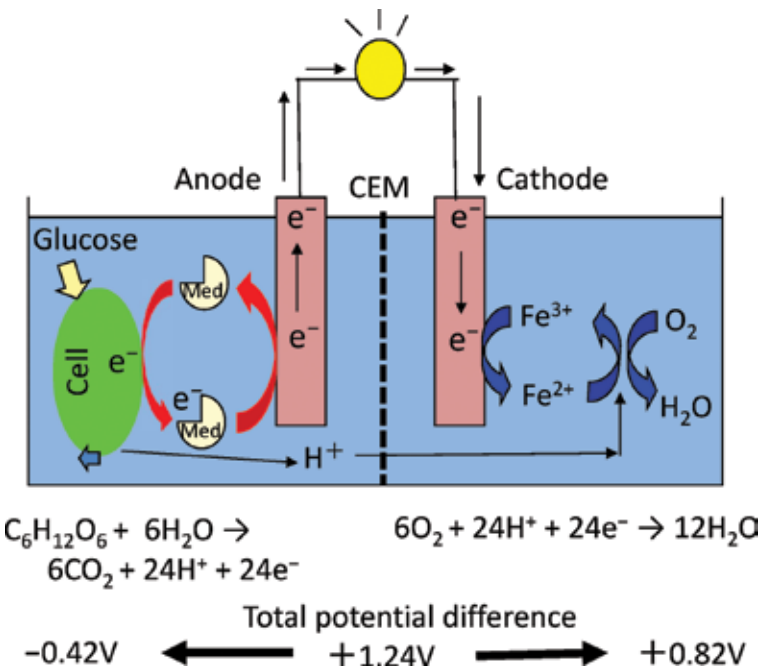

Figure 4.
General dual-type MFC. Med: mediator, CEM: cation exchange membrane.

because it significantly regulates the movement of the protons responsible for the pH reduction at the anode affecting the activity of microorganisms and the delivery of electrons to the oxygen at the cathode. Also, some factors to consider, such as durability and cost, are important for selecting CEM. At present, Nafion is popular for many MFCs [30, 31].

Numerous research studies are being conducted to evaluate the influence of the electrode materials on the performance and cost of the MFCs. Carbon materials, which are noncorrosive, have been widely used because of their high electrical conductivity and chemical stability, e.g., carbon rod, carbon fiber, carbon felt, and carbon cloth [3]. Biocompatibility, specific surface area, electrical conductivity, and cost are important factors for its selection. Since its discovery in 2004, graphene has been attracting much attention for its use as an electrode because of its high specific surface area, electrical conductivity, and biocompatibility [32]. In fact, graphene has been already used in lithium-ion batteries, and the development of graphene-modified materials to increase the power density has progressed actively [33, 34]. Moreover, since biofilm formation by microorganisms on the electrodes affects the performance of MFCs, the preference of electrode materials tends to shift from two-dimensional to three-dimensional surfaces, where a larger surface area is obtained; thus, the contact with microorganisms increases. Furthermore, metals are also used as the electrodes. The conductivities are higher than those of carbon materials, but they are prone to corrosion in the anode solution. Therefore, metals are problematic to use, except for stainless and titanium. To improve such problem, materials in which metal is incorporated into graphite have been made [3].

A phosphate buffer or bicarbonate buffer solution is often used for the anode electrode solution to achieve high performance [16, 35]. The pH of the solution affects not only the activity of microorganisms but also the transfer of hydrogen ions used from the anode to the cathode when the electrons are transferred to oxygen at the cathode. The solutions contain microorganisms as the catalyst, organic matter as the fuel, and mediator as the electron carrier. In addition, there are reports that the performance of MFCs was improved by adding NaCl to increase the ionic strength [36].

Regarding the fuel, many substrates have already been studied [37]. For example, acetic acid, lactic acid, glycerol, glucose, xylose, sucrose, starch, yeast extract, malt extract, various real wastewaters, and synthetic wastewater were used depending on the purpose of each research. Generally, the fermentable substrate of microorganisms is used to generate electricity more efficiently. There is a trend where glucose is used when using *S. cerevisiae*, lactic acid when using *S. oneidensis*, and acetate when using *G. sulfurreducens* in the experiments. On the basis of a calculation, when lactic acid, acetic acid, and glycerol are completely oxidized, there are 14, 8, and 14 electrons obtained, respectively. The number of electrons obtained from each substrate depends on the metabolic pathway.

Regarding the mediator, although some microorganisms can carry electrons directly to the electrode as described later, in many cases, the electrons cannot be carried, or the performance is low even if carried, so an artificial mediator that can pass through the cell membrane is added to the anode solution. The typical compounds for artificial mediators are methylene blue, neutral red, 2-hydroxy-1,4-naphthoquinone (HNQ), thionine, benzyl viologen, 2,6-dichlorophenolindophenol, and various phenazines [38]. It was reported that the hydrogenase donates electrons to the neutral red [39], but the process was not yet clearly proven as to how these mediators deprive electrons of the cell. It is thought that, depending on the type of mediator, the electrons may be taken directly from NADH or obtained from the electron transfer system of the cell membrane. On the other hand, there is also a difficult aspect of using a mediator. In order to increase the electron transfer

efficiency by the mediator, it requires a high concentration, but because of its high toxicity, it has a strong influence on the cells; therefore, the level of use is necessary to be controlled.

Finally, the cathode solution is explained as follows. The electrons generated at the anode are carried to the cathode, where the reduction reaction takes place. When oxygen, the most common electron acceptor, is used as an oxidizing agent, aeration is necessary because oxygen has low solubility (about 8 mg/L DO). There are cases where oxygen generation by the photosynthesis of algae is used for oxygen supply [40, 41]. In the reaction at the cathode, H_2O is produced by oxygen, whereby the electrons were carried from the anode via an external circuit and protons were carried from the anode solution via CEM. There is also a report that the addition of hydrogen peroxide leads to an improvement in power generation [42]. Besides oxygen, there are various electron acceptors [43]; for example, an oxidizing agent such as iron ferricyanide is also used for the cathode. In many cases, the ferricyanide has a high mass transfer efficiency and a high cathode potential so that a high output can be obtained. The combination of carbon electrodes and ferricyanides to achieve power 50–80% higher than the combination of Pt/carbon electrodes and oxygen was reported [44]. In the case of using ferricyanide, once the trivalent iron ion receives the electrons, it becomes divalent, and when it delivers the electrons to oxygen, it reverts to the trivalent state. However, the latter reaction is less likely to occur owing to the low solubility of oxygen. Ferricyanide is an excellent electron acceptor, but owing to its toxicity, its use is generally limited to the laboratory. Other than oxygen and ferricyanide, there are also many candidates, for example, nitrate, persulfate, permanganate, and manganese dioxide. It is also possible to use the nitrate contained in the wastewater because its redox potential is close to that of oxygen, and then, the nitrate is reduced to nitrogen gas at the cathode [43].

2.4 Other types of MFCs

MFCs are typically divided into a dual-chambered cell described above and a single-chambered cell (**Figure 5**). In the latter, a membrane-type positive electrode with oxygen permeability called an air cathode is used [45]. The electrode is coated with the platinum catalyst, and H_2O is produced from the oxygen permeated from the atmosphere, the proton in solution, and the electron from the anode. However,

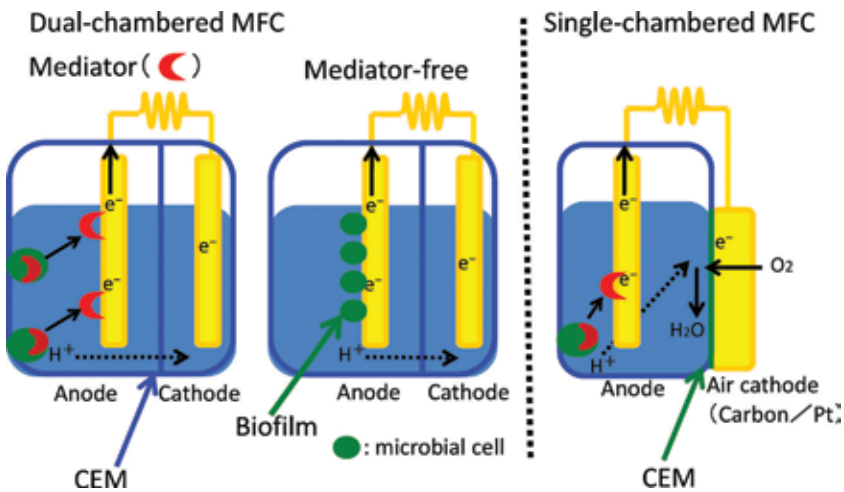

Figure 5.
Other types of MFCs. Med: mediator, CEM: cation exchange membrane.

if the permeated excess oxygen diffuses and is delivered to the microorganisms at the anode, the electrons generated in the microorganisms are then transferred to the oxygen, and the energy recovery rate decreases. Therefore, a CEM is used between the anode solution and the cathode in order to prevent a decrease in energy recovery. This single-chambered type has already been widely used currently.

Moreover, there is a mediator-free type that does not require an artificial electron compound [46]. The microbial strains used here can synthesize mediators themselves and/or have an electron transfer function on the cell surface. In the former, the self-synthesized mediators are flavin compounds, hydroquinone, and phenazine that are able to transfer the electrons to the electrode. In the latter, membrane-bound proteins such as pili, c-type cytochromes, and filaments are known as cell surface structures that can directly transmit electrons. The biofilm formation on the electrodes, namely, biocompatibility of the electrodes, is also important for power generation via such direct electron transfer. Therefore, research on electrodes promoting the formation of the biofilm is actively being conducted.

In addition, with the use of MFCs in wastewater treatment, contamination on the CEM results in reduced power generation; hence, membrane-free MFCs have also been studied [46].

2.5 Various microbial catalysts

Various microorganisms have been studied for a long time since the first experiments on *S. cerevisiae* and *E. coli* [4]. The classification of these catalysts is largely based on the purity and complexity of the cultured microbial systems. Many different microorganisms are used in the pure system [37, 38, 47, 48]. *S. cerevisiae* is a safe microorganism used in foods and can grow even in the presence of a high concentration of sugar, sulfate, and ammonium nitrogen. MFCs show high performance when using *S. cerevisiae* and glucose as a catalyst and a fuel, respectively [43]. *E. coli* can also ferment sugar well and is used for the study of MFCs using glucose as a fuel. Although it can generate electricity without a mediator, in the present situation, the power generated is low, so an artificial mediator is added in order to achieve better performance. Besides the two examples, there are also *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Rhodoferax ferrireducens*, *Geothrix fermentans*, *Shewanella* species, *Geobacter* species, *Clostridium* species, and sulfate-reducing bacteria. The possibility of utilizing extremophilic microorganisms is also being studied [49], and to add a new perspective to power generation by MFCs, the utilization of photosynthetic bacteria at the anode is also examined [40]. One of the advantages of these MFCs is the elimination of carbon dioxide released into the atmosphere. Meanwhile, in complex systems, the use of various wastewater and waste sludge has been reported [25, 37, 46, 50]. Many studies on bacterial communities under the control of MFCs have been conducted using those aforementioned resources. It is thought that the bacteria belonging to the phylum Proteobacteria were involved in power generation [51, 52]. However, owing to the complexity of bacterial interactions, their contribution to power generation within these communities is not well understood yet.

In such a research situation, there are relatively many examples of research on *S. oneidensis* and *G. sulfurreducens*, and the details of their power generation mechanisms are being clarified. *S. oneidensis* can produce self-synthesized mediators, like flavin compounds. The strain has not only such a mediator but also an extracellular electron transport system involved in power generation. This system, present from the inner membrane to the outer membrane, plays a role in carrying the electrons to the extracellular receptors (i.e., the electrodes in this case) by contacting them directly. In particular, cell-surface-localized cytochromes (MtrC and OmcA) are

important components for the electron transfer [53]. On the other hand, *G. sulfurreducens* has electrically conductive pili, called nanowires, which can transfer electrons to extracellular electron acceptors on the cell surface [54]. *S. oneidensis* also has an electrically conductive structure similar to the pili, but its structure is different, whereby the membrane structure containing the cytochrome protein described above was raised [54]. In any case, it has been confirmed that electrons can be delivered via such protrusions. It is expected that new developments will be made in the future, such as introducing the genes related to these mechanisms into other species, especially model organisms, such as *E. coli* and *S. cerevisiae*.

3. Recent topics of microbial catalyst and future directions

3.1 Modification of microorganisms

The utilization of chemical and biotechnological techniques is important to modify the microbial cells as biocatalysts in the MFC system. Molecular biology approaches are effective tools to improve the performance of the biocatalysts for the desired system. In this section, recent topics about the approaches for microbial catalyst development are discussed.

3.1.1 Chemical treatment

Mediators and macromolecular catabolic enzymes, which are used for electron transfer and other metabolic activities, are abundant in the cytoplasm of the microbial cells used in the MFCs. However, it is not easy to transport the mediator molecules to the bacterial outer membrane so as to reach the electrode. The lipopolysaccharide (LPS) layer on the Gram-negative outer membrane is compact and nonconductive; thus, most microbial cells are nonconductive [55]. It was found that chemically perforated pores and channels on the cell membranes accelerated electron transfer, leading to an improved power output for an MFC using *P. aeruginosa* [56]. In their study, polyethyleneimine (PEI)-treated biofilm achieved a doubled power density (41 mW/m^2) compared to the control biofilms. The large pores and channels on the cell membrane created by PEI treatment promoted the diffusion of the self-produced mediators (pyocyanin and pyorubin) of *P. aeruginosa*. The modified cell membrane surface also promoted the adherence of microbial cells to the electrode, which further improved the electron transfer. This method was also applied to *E. coli* [57]. Recently, it has been reported that lysozyme treatment increased 1.75-fold of the MFC performance with *K. rhizophila* P2-A-5 [58]. Thus, chemical treatment is one of the important approaches to modify the microbial cells for the improvement of the MFC performance.

3.1.2 Biosurfactant production by gene modification

To increase the cell permeability of biocatalysts in the MFCs, Zheng et al. proposed a new approach by inducing the biosurfactant production based on a genetic modification [59]. It is true that the efficiency of membrane permeability can be improved with a biosurfactant, which ultimately increases the transport across the membrane. In addition, overexpression of the *rhlA* gene, which is responsible for rhamnolipid (a biosurfactant) production, was also conducted [60]. The biosurfactant directly influenced the overproduction of rhamnolipids from the electrical bacteria, such as *P. aeruginosa*. The electron transport across the membrane was greatly increased as the membrane permeability increased. The power output of the

MFC-catalyzed process by the biosurfactant-producing bacterium was enhanced up to about 2.5 times compared with the wild type.

3.1.3 Introducing the extracellular electron transfer pathway

The sparse availability of genetic tools in manipulating electricity-generating bacteria and the multiple overlapping pathways for extracellular electron transfer make it challenging to modulate electron transfer and/or introduce other functions of interest. In response to this challenge, several studies have taken the complementary approach of engineering portions of the extracellular electron transfer pathways into the well-studied industrial microbe *E. coli* [61]. In these studies, MtrCAB of *S. oneidensis* was successfully expressed in the *E. coli* cells, and the activity of these proteins was confirmed by the metal reduction. Although the introduction of MtrCAB permits extracellular electron transfer in *E. coli*, the low electron flux and the absence of growth in these cells limit their practical application. Recently, in addition to surface-localized cytochromes, it has been further confirmed that CymA, the inner membrane component of *S. oneidensis*, significantly improved the extracellular electron transfer rate or cell viability. This recombinant *E. coli* achieved current generation in an MFC system without the addition of mediators [62, 63]. Our research group is trying to develop an excellent *E. coli* biocatalyst for the anode in an MFC system based on the combination of engineering of central metabolism and introduction of extracellular electron transfer in the presence of an HNQ mediator.

3.2 Extremophilic microorganisms

An extremophilic microorganism thrives in physically or geochemically extreme conditions that are detrimental to most life on Earth. They thrive in extreme hot niches, ice, and salt solutions, as well as acid and alkaline conditions; some may grow in toxic waste, organic solvents, heavy metals, or several other habitats that were previously considered inhospitable for life. Extremophiles can be used to oxidize sulfur compounds in acidic pH to remediate wastewaters and generate electrical energy from marine sediment microbial fuel cells at low temperatures. The MFC performance of these extremophilic microorganisms has been well summarized in several review papers [49, 64]. In this section, the recent advances of MFCs using extremophilic microorganisms as catalysts are briefly introduced and discussed.

3.2.1 Acidophiles and alkaliphiles

An increase of cell voltage is seen at increasing anode pH because of the additional pH gradient representing a source of energy. The practical implication of an elevated cell voltage is that more energy can be gained from MFC systems at higher pH values. By contrast, operating the anode of MFCs at an acidic condition has an advantage that the protons will not cause diffusion limitations in the cathode compartment for the reduction of oxygen, and therefore, it will not limit the current production [65]. However, under a low-pH condition, the microbial cells have to maintain a near-neutral cytoplasm [66] which consumes a portion of the energy derived from the electron transport for other processes, such as proton export, that increases the anode overpotential, leading to decreasing power generation. At pH 2.5, *Acidiphilium* sp. isolated from the environment mediates a direct electron transfer from the glucose metabolism to the anode at a rate of 3 A/m^2 even in the presence of air [67]. This interesting strain produces extracellular polymeric substances and forms a biofilm between the carbon microfibers and in pores on the graphite rod surface [68].

Meanwhile, at high pH, the lower anode potential results in an increased cell voltage. In general, the anode becomes acidified during the MFC operation, and the cathode becomes more alkaline, followed by a reduced cell voltage and power output. *Pseudomonas alcaliphila* is capable of electricity production at high pH, excreting phenazine-1-carboxylic acid that acts as an electron shuttle during the oxidation of citrate [69]. An MFC has also been developed to treat food wastes that comprise 30–55% of all refuse in urban societies. The waste was first treated by anaerobic digestion, the resulting food waste leachate was amended with 100 mM NaCl, and then, electricity was generated in a pH 9 MFC that had a maximum of 63% Coulombic efficiency [70]. Recently, a tubular upflow MFC utilizing seafood processing wastewater has achieved a maximum power density of 105 mW/m^2 at pH 9 [71].

3.2.2 Psychrophiles and thermophiles

Temperature also has a major impact on the output of MFCs. It influences the activity of microorganisms, the electrochemical reactions, and the Gibbs free energy change of the reactions. There exists an optimum temperature for enzymes in the MFCs, and the electrochemical reaction rate increases with increased temperatures. A lower operating temperature adversely influences the output, start-up time, and substrate oxidation rate in the MFC system. This negatively impacts the MFCs for processes such as wastewater treatment because the streams are generally at low temperatures. However, the advantage of low temperature for the MFCs is that they typically produce higher Coulombic efficiencies [72, 73]. The microbial community was enriched from the anaerobic sludge at the anode of an acetate-fed MFC operated at 15°C with psychrophiles *Simplicispira* sp. and *Geobacter* sp. [74]. Another study at 5–10°C enriched the low-temperature microorganisms from the genera *Arcobacter*, *Pseudomonas*, and *Geobacter* [75]. One promising application of the low-temperature MFCs is that for low-power-consuming devices like sensors that are intended to last for an extended period of time.

On the other hand, the advantages of operating at high temperatures are higher microbial activity, better substrate solubility, high mass transfer rate, and lower contamination risk. An example of an improved current generation at a high temperature (60°C) is a marine sediment MFC that generated 209–254 mA/m^2 compared with 10–22 mA/m^2 at 22°C [76]. Recently, an MFC with a higher operating temperature (70°C) has generated 6800 mA/m^2 [77]. Furthermore, the hyperthermophilic MFCs were operated at above 80°C [78]. However, a negative point of thermophilic MFCs is higher rates of evaporation than the MFC system itself. Therefore, a continuous mode of MFCs was proposed to allow a replacement of the anolyte and catholyte [79].

In this section, recent topics of microbial catalysts for MFCs were introduced. There are two approaches in developing the microbial catalysts. One is the modification of existing microorganisms using chemical treatments or biotechnological techniques, including gene editing. The other is exploring new microorganisms from the environment, including extreme conditions. Although new findings and knowledge were obtained from both approaches, a drastic improvement on the MFC performance to achieve a paradigm shift has not appeared yet. In parallel with the improvement of microbial catalysts, the development of the fuel cell system, including the electrodes, was intensively studied to increase the output of MFCs. In particular, the application of graphene-modified electrodes [33] and the investigation of electron acceptors [43] have shown remarkable progress in the past decade. In order to actualize the practical use of MFCs, a synergistic impact from the combination of microbial catalyst and fuel cell system is essential.

4. Conclusion

This chapter focuses on the significance of MFC development, the historical background and fundamental principles of MFCs, and their recent developments, especially on microbial catalysts. MFCs have not reached the desirable level of power generation that supports daily life because of the problems such as scaling-up. On the other hand, developments of technology combining wastewater treatment and power generation, and application for environmental sensors are progressing to a stage close to practical use. If these popularizations continue, it will further develop its application in broader fields. Owing to their limitations, it may be difficult to force MFCs to become the main power supply in our daily life in the future, but it seems possible to use them as an auxiliary power supply. In addition, MFCs may become useful as a power supply in areas where the infrastructure is not well developed, for example, a portable power supply generating electricity if water is added. Regarding microbial catalysts, it is also known that various microorganisms can generate electricity, and if this superior power-generating function of these microorganisms can be integrated into a microbial cell using the synthetic biological method developed recently, the ability of the microbial catalyst will dramatically increase. Soon, its power generation ability could be greatly improved in combination with the progress of other constituents.

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Conflict of interest

The authors declare no conflicts of interest associated with this text.

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Chapter 5

Integrated Biologics Manufacturing in Stirred-Suspension Bioreactor: A Stem Cell Perspective

Suman C. Nath and Derrick E. Rancourt

Abstract

Stem cell therapy is garnering attention as several clinical trials have taken place in the recent years by using human pluripotent stem cells (hPSCs). Hundreds of biotechnological companies are investing to find a permanent cure for difficult-to-treat diseases like age-related macular degeneration, Parkinson's disease, diabetes, etc. by using hPSCs. Therefore, clinical-grade cell manufacturing has become an important issue to make cell therapy products safe and effective. Current manufacturing practices are adopted from conventional antibody or protein production in the pharmaceutical industry where cells are used as a vector for producing the desired products. In cell therapy applications, cells are the products that are sensitive to physicochemical parameters and storage conditions anywhere between isolation to patient administration. Moreover, cell-based product manufacturing consists of multi-step processing, including isolation from patients, genetic modification, derivation, expansion, differentiation, purification, characterization, cryopreservation, etc. This can require long processing times and pose high risk of product contamination as well as high production cost. Herein, we discuss the current methods of biologics manufacturing and its limitations. We also review current practices for integrating and automating cell manufacturing facilities. Finally, we propose how to integrate multi-step cell processing in a single bioreactor to make the cell manufacturing practices more direct.

Keywords: biologics, stem cell therapy, genetic modification, integrated manufacturing, bioreactor

1. Introduction

Based on their self-renewal and differentiation capabilities, human pluripotent stem cells (hPSCs) including embryonic stem cells (ESCs) [1] and induced pluripotent stem cells (iPSCs) [2] are attractive tools in the field of regenerative medicine. After the discovery of hiPSCs in 2007, this field expanded vigorously and hundreds of biotechnological companies were established to use these cells for treating degenerative diseases. The most common degenerative diseases treated by the hESCs are age-related macular degeneration (AMD), type I diabetes mellitus, heart failure, Parkinson's disease, and spinal cord injury [3]. Although hiPSCs are a better source

for autologous cell therapy applications, they are less preferable for clinical trials because of less genetic stability compared to the hESCs. However, a few clinical trials have already been started using the patient-derived hiPSCs. The Takahashi group from the Riken Center for Developmental Biology has recently conducted a clinical trial for treating wet AMD [4]. Similarly, a Takahashi from Kyoto University is conducting a clinical trial for treating Parkinson's disease by using hiPSCs [5]. A few clinical trials are also ongoing in the USA for treating different diseases like β-thalassemia, liver diseases, diabetes, etc. using hiPSCs and their use is expanding worldwide day by day [6].

As stem cell therapy is garnering increasing attention, a lot of clinical trials are ongoing using both hESCs and hiPSCs cells. About 6849 clinical trials and 1415 stem cell-based therapies were found based upon searches we recently performed on clinicaltrials.gov (October, 2018) [7]. However, the percentage of success is not high enough as speculated from the previous clinical trials. Among the 315 clinical trials conducted (26.0% Phase 1, 40.6% Phase 1/2, 22.5% Phase 2, 3.8% Phase 2/3, and 6.7% Phase 3), only 0.3% went to Phase 4 [3]. The low percentage of completion of clinical trials depends on various factors. One of the major factors is manufacturing practices that can provide high safety and efficacy of cell therapy products. Moreover, production cost of multiple doses also hinders the success rate of clinical trials. As cell therapy revenue exceeded multi-million dollars and has been a profitable business in recent years, but much attention is needed to produce high quality cells for treating incurable diseases [8, 9].

The production of stem cell biologics is adapted from the conventional pharmaceutical protein and vaccine production. Conventional biologics production involves the following basic steps: isolation and identification of raw materials, formulation, filling, packaging, and storage, where the total processing stops at the storage of final products.

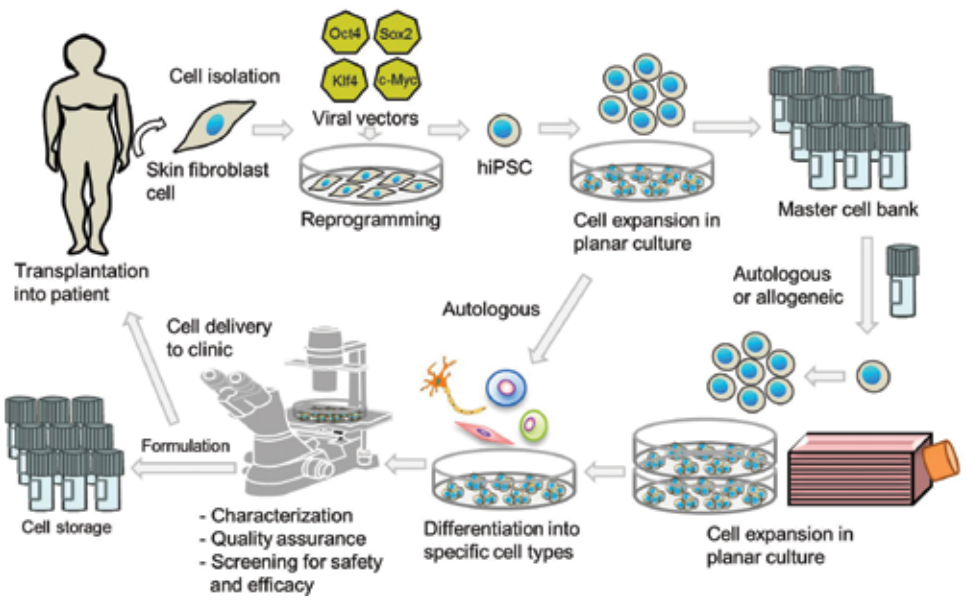

Figure 1.
Schematic illustration of current multi-step cell manufacturing strategies in planar culture for stem cell therapy applications. Skin cells are isolated from the patient and reprogrammed to hiPSCs using viral vectors. After reprogramming, hiPSCs are stored in a master cell bank or differentiated directly in autologous cell therapy applications. In some cases like allogeneic cell therapy applications, cells are expanded in a large amount and then differentiated. After performing characterization, quality assurance, and screening for safety and efficacy, cells are delivered to hospital or stored in a cell bank for future use.

There is a big difference between the production of conventional biologics and cell-based therapy products. For vaccines or pharmaceutical protein production, cells are used as a platform for obtaining desired proteins. After that, cells are discarded. However, in cell-based therapies, cells, which are sensitive to the physical or chemical attributes of the residing environment, are the final products. Therefore, much consideration is needed before translating cell-based products from bench to clinic. This extends to the acquisition of tissue samples and isolation of cells, initial cell purification, selection, activation and transduction, cell expansion in plate or bioreactor culture, differentiation, washing, harvesting and formulation, filling and cryopreservation, and finally, storage and delivery to the clinics (**Figure 1**) [10].

Cumbersome multi-step manufacturing systems can cause batch to batch variability, inefficacy, and low quality of cells for transplantation and need to be simplified and made more direct. In this context, we will discuss current limitations of cell manufacturing strategies and propose how to overcome these by integrating the total process in a single bioreactor to make cell manufacturing straight forward enough to deliver high quality cell therapy products to the clinic. In this review, we will also discuss how to integrate genetic modification—transfection or transduction, reprogramming, differentiation, purification, and formulation of final products in a single bioreactor.

2. Current manufacturing strategies for stem cell therapy

Current manufacturing strategies for cell therapy products are replicated from biologics manufacturing in the pharmaceutical industry. However, the processing of cells is far different from pharmaceutical proteins or vaccines. For pharmaceutical peptide production from microorganisms, the raw materials are extracted from bacteria or fungus [11, 12]. They are then separated, purified, and examined for quality assurance to meet the requirements of regulatory agencies, e.g., Food and Drug Administration (FDA), British Pharmacopeia, etc. The final products are stored or marketed in a dose-dependent manner.

Cell processing is more intensified when the pharmaceutical proteins are produced by using human, animal, or plant cells as a by-product. In this case, high quality products depend on the maintenance of high quality cells, and maintaining a sterile condition is very important. Therefore, good bioprocessing is required to optimize the production of desired proteins. After inoculating from a master cell bank, the cells are cultured for a specific period of time [13, 14]. The supernatant is then collected and the desired proteins are separated, purified, and concentrated. The isolated products then go through quality assurance to meet the criteria of the regulatory agency. Finally, the products are stored and marketed in a dose-dependent manner.

The manufacturing of stem-cell based products is not as straight forward as the production of pharmaceutical proteins or vaccines. This is because cells are the final product in stem cell therapy and are vulnerable to physical or chemical operations from isolation to delivery to patients. Cell manufacturing strategies also vary from source to source and depend on autologous or allogeneic transplantation (**Figure 1**). The major general steps are the acquisition of tissue samples and isolation of cells, initial cell purification, selection, activation and transduction, cell expansion, differentiation, washing, harvesting and formulation, filling and cryopreservation, and finally, storage and delivery to the clinics [10].

For stem-cell based products, cells are isolated from specific tissues of patients, e.g., blood, skin, etc. for autologous transplantation or can be used from cell banks for allogeneic transplantation. Heterogeneity of final products may arise from the cell isolation step because patients' tissues contain various undesired

subpopulations. For example, in chimeric antigen receptor T-cell (CAR-T) therapy, cells are isolated from patients' blood tissue, which contains abnormal levels of inhibitory factors and regulatory cells [15, 16] because patients are treated with chemo- and radiotherapies. As a result, heterogeneity occurs in the final products, which need much attention during the cell isolation step. Cells isolated from patients need to be purified by centrifugation, magnetic-activated cell sorting (MACS), or fluorescent-activated cell sorting (FACS). Then, initial cell culture is done for selection, activation, or transduction of specific interest.

After purification, cells are expanded in plate culture or bioreactor. Based on demand, large-scale expansion is required in a sterile condition, which also requires intensive consideration because it is the rate-limiting step for commercialization of cell therapy products. The most important considerations for large-scale expansions are: operational, economic, quality and safety.

Operational design for culture systems (2D or 3D) with manual or automatic (desirable) operation is important before large-scale expansion [17]. Bioreactors are superior to plate culture for obtaining a large number of cells. Online monitoring and control of process parameters (pH, DO, pCO_2, etc.) and considering the shortest possible culture time are also important parameters for operational consideration. A prediction model for medium consumption (glucose and glutamine) and toxic material production (lactic acid and ammonium) is very useful for determining medium feeding regimen. A dedicated single-use vessel is also a big operational consideration before large-scale expansion of cell-based products.

As cell-based products are costlier, economic considerations for medium, efficient cell lines and other indirect utilities are important. However, the most important consideration in large-scale expansion is product quality and safety. For this purpose, dedicated cell manufacturing facilities are required to maintain current manufacturing practices (cGMP) for high product purity and safety.

After large-scale expansion, cells are harvested by detaching them from the culture substrate using enzymatic treatment. Non-enzymatic detachment is also available by changing temperature or pH [18–20]. Aggregate culture in bioreactors may not necessarily need a detachment step for harvesting [21–25]. Next steps are washing and volume reduction, which can be done by centrifugation or tangential flow filtration on a large scale by using automated commercial devices (kSep systems and Terumo BCT).

Purified cells are formulated in a dose-dependent manner and checked for quality assurance. Quality assurance is done in three different stages: microbial contamination, chemical contamination, and quality or potency assurance. Microbial contamination is checked for bacterial, fungal, or viral contamination by sterility tests with various methods [26, 27]. The most commonly used sterility test is a 14-day incubation of cell products for bacterial and fungal contamination [28, 29]. Chemical testing includes checking for molecules accompanying the culture medium or other factors used during isolation, expansion, and storage. One commonly used chemical test is the LAL test for bacterial endotoxin. There is now an automated 15 min test for determining endotoxin in cell therapy products, which was developed following FDA regulations [30]. Other chemical testing concerns are checking for residual proteins of different origins, serum, and other harmful particles originated from cell processing.

In cell therapy products, quality is the major concern, especially because cell growth is a requirement. For that reason, a cell viability assay is done to determine live or dead cells in the product using a variety of staining methods. Colony forming unit (CFU) is also useful for determining biological activity of cell therapy products [31, 32]. Product potency is an important criterion to meet before releasing the product. For example, if a cell therapy product is applied for the chimeric antigen

receptor T (CART)-related cancer therapy, it needs to be examined for the secretion of cytotoxic cytokines (IFN-γ) and killing of target cells [33]. However, for hPSCs, the final products are differentiated cells, wherein potency should be checked via transplantation into disease models.

For hPSC-derived products, strict quality control is imperative before transplantation to the patients because there is high risk of oncogene transfer to patients. A clinical trial was halted in 2015 in Japan while treating AMD by autologous hiPSC-derived retinal pigmented epithelial cells because of genetic abnormality [34]. Since genetic abnormalities occur in hiPSC-derived products from reprogramming to finally differentiated cells [35], cells should be strictly screened for epigenetic signatures, karyotyping, telomerase activity, mitochondrial remodeling, etc. [36–38]. Rohani et al. summarized possible molecular cytogenetics for quality control that should be checked before releasing the final products [39]. Some of the proposed quality testings are whole-genome sequencing, single-cell genome sequencing, epigenomic analysis, and mitochondrial DNA integrity testing for maximizing the patient safety.

After passing the product quality assurance, cells need to be delivered to clinics immediately or stored for future use. Cells are shipped generally to the clinics on dry ice (−78°C) or in liquid nitrogen dry shippers (−160°C) if the cells are vitrified. The mostly used technique for cell storage is cryopreservation in liquid nitrogen at −196°C which is adapted from the conventional stem cell banking [40, 41]. For cryopreservation, dimethyl sulfoxide (DMSO), glycerol, sugars, or other polymers are used. Among them, clinical grade DMSO is widely used although it is detrimental and can cause harmful effects to cells [42, 43]. Therefore, removing it from cryopreservation protocols or lowering the concentration is important. However, developing appropriate protocols for freezing and thawing is also important for high recovery of cells. Generally, slow-freezing and quick thawing is highly applicable for better recovery of cryopreserved cells [44, 45]. Since intracellular ice crystal formation is a big obstacle in cryopreservation, using ice recrystallization inhibitors is also an effective process for cryopreservation of clinical cell therapy products [46, 47].

Product delivery is also an important step to consider before administration to the patients. Since the products are carried in an environment where temperature is extreme, the container should be made with such materials that can withstand extreme low temperature and do not cause any leakage compromising the product quality [48]. For autologous cell therapy applications especially for CAR-T cell therapy, a dedicated vessel, which can withstand extreme low temperature, is needed [49].

3. Integrated biologics manufacturing in bioreactors

The conventional production of pharmaceutical proteins or other biologics consists of multiple steps from raw materials to finished products. As biologics need to maintain stringent quality control, multiple steps in production facilities compromise the product quality significantly. They also reduce productivity and become prone to human errors, which decrease product efficacy and safety. Moreover, multiple steps in cell processing consume a lot of time, which indirectly increases production cost. To overcome these drawbacks, integrated pharmaceutical production has been attempted by various pharmaceutical companies. One of the significant attempts was made by the Novartis-MIT Center for Continuous Manufacturing of pharmaceutical products to fully integrate the cell processing system [50, 51]. Another attempt was taken by Genzyme™ for continuous production of pharmaceutical recombinant protein in bioreactors, where cell culture to product isolation and purification was integrated in a single flow [52]. By using this system,

they respectively reported successful production of monoclonal antibody as well as highly complex, less stable pharmaceutical protein with consistent product quality, high product output, and low cost. Process integrity is necessary for reducing cumbersome production steps and cutting cost significantly. One such integrated system developed by Johnson & Johnson has recently got FDA approval for large-scale HIV drug production [53] that reduces time and cost by one third compared to the conventional batch processing.

Since biologics production for cell therapies require multiple steps, integration of all of the steps will give high product quality and safety, as well as help overcome stringent regulatory requirements. In this context, we will discuss how to integrate some important basic steps of cell manufacturing especially genetic modification, cellular reprogramming, expansion, and differentiation in bioreactors to promote a single-step approach for cell-based therapies (**Figure 2**).

3.1 Genetic modifications in bioreactor

Genetic modification is one of the biggest steps in producing cell therapy products. In biologics manufacturing, it has been practiced for many years for producing antibodies, proteins, or other biotechnological drugs. It has also been used extensively in the cell therapy industry as various cell-based products have been applied for treating multiple incurable genetic diseases in recent years. Some genetic modifications affect patients directly and some indirectly. For example, in adrenoleukodystrophy (ALD), a neurological disorder occurs due to malfunction of oligodendrocytes and microglia where genetic modification can affect a patient directly. To recover from it, a corrected gene is inserted into the patient-derived hPSCs and transplanted into the

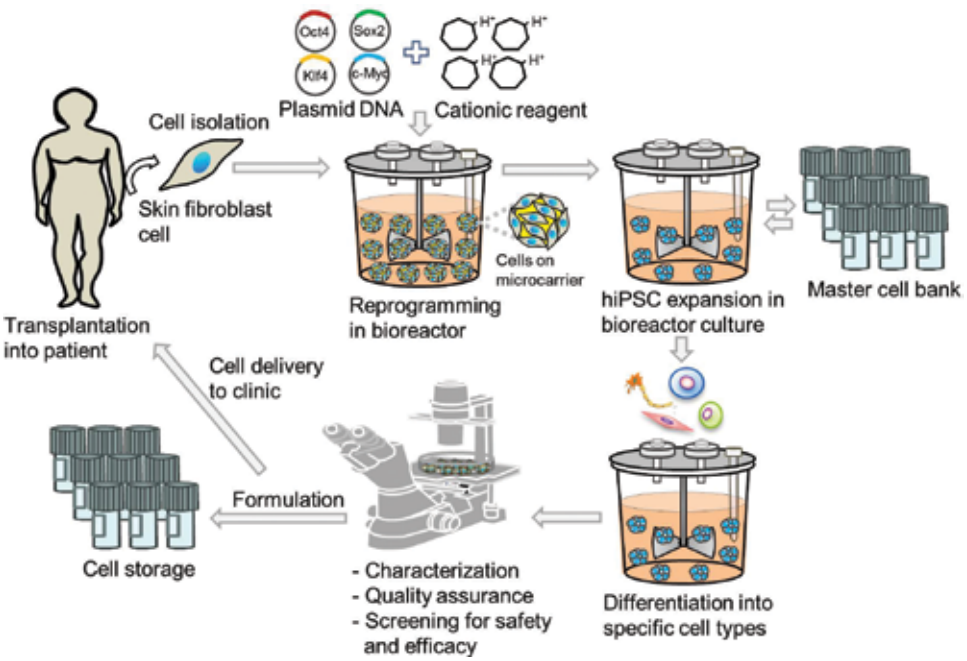

Figure 2.
Schematic illustrations of integrated single-step cell manufacturing strategies in bioreactor culture for stem cell therapy applications. Skin cells are isolated from the patient and reprogrammed to hiPSCs on microcarriers using a nonviral approach. After expansion as aggregates, hiPSCs are stored in a master cell bank or differentiated directly in bioreactor. After performing characterization, quality assurance, and screening for safety and efficacy, cells are delivered to hospital or stored in a cell bank for future use.

patient's brain, which is differentiated into microglia to promote production of myelin in the patient's brain that recovered the ALD [54].

In some gene therapy applications, patients are exposed indirectly to genetic modification. For example, in thalassemia, patient blood cells are extracted from the body and the cells are modified and enriched in *ex vivo* to target the specific antigens of patients' body [55]. Other indirect genetic modifications used for treating CAR or T-cell receptor (TCR) genes to T-cells [56], expression of CD40 ligand in dendritic cells [57], adenosine-deaminase severe-combined immunodeficiency [58], and beta-thalassemia [59], as well as deletion or insertion of desired genes in a specific genomic location. Among them, CAR-T cell therapy has got much attention for treating cancer-related diseases. These genetically modified T-cells can specifically target the antigens and kill the cancer cells efficiently [60]. CARs and TCRs are the mostly used receptors which are engineered to activate the T-cells [61]. Nowadays, a lot of CAR-T cell-based therapies are being established for treating advanced-stage lymphoma [62] and B-cell lymphoma [63] as well as other autoimmune diseases [64].

Viral vectors are commonly used to deliver genetic cargo to cells (**Figure 1**). This involves a two-step process: preparation for viral vectors and transduction for modifying the cells to express desired property. Lentiviral and gamma-retroviral are widely-used for their superior transduction efficiency but their transgenes are integrated with the host genome [65]. Another choice for viral transduction is adenovirus where viral transgenes are not integrated into the host genome but less efficient than lenti- and retro-virus. The major drawbacks in viral vector mediated transduction are concerns for safety of the products [66]. Viral vectors are widely used for reprogramming hiPSCs from skin fibroblasts cells [2].

Other methods for cellular transduction use nonviral approaches, including nucleofection or electroporation, or liposome-mediated delivery of DNA or RNA into cells. Although DNA vectors are easy to scale-up, carry large-size DNA with less immunotoxicity, this process is less efficient than the viral transduction. There are some other methods for skipping the use of viral vectors which are also efficient in doing the transgene expression [67–69]. Hsu et al. reported successful transfection by using commercially available nonviral cationic reagents, for example, TransIT-3D, TransIT-2020, XtremeGENE 9, XtremeGENE HP, JetPrime, Lipofectamine 3000, and Effectene and compared their transfection efficiency [70]. Warren et al. reported efficient reprogramming of hiPSCs from various cell sources by using mRNA and differentiated the cells into three germ layers [71]. hiPSCs were also reprogrammed by using recombinant protein that also maintained all the three germ layers [72].

Since transgene possesses high risk of cancer-causing agents; therefore, removal after transduction is highly desired. There are a few methods developed for the removal of these vectors. One of the methods is the piggyBac transposon system, which has been used to remove tandem Yamanaka reprograming genes Oct4, Sox2, Klf4, and c-Myc from iPSCs following reprogramming [73]. Removal of transgenes after incorporating CAR into T-cells used another transposon system called Sleeping Beauty, which successfully removed any genetic scar from the transduced cells [74, 75]. Likewise, transgene-free iPSCs have also been produced by Cre excision of reprogramming genes via loxP sites [76]. Integration-deficient viral vectors are also good candidates for producing transgene-free cell therapy products by mutating viral integrase [77]. Another approach is to use site-directed integration using targeting nucleases [78–80].

Various genome engineering technologies have been explored for gene addition, deletion, or correction in the cell therapy industry and are increasing day by day [81]. The most widely used targeting nucleases are zinc-finger nucleases (ZFNs), clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas

endonucleases, or transcription-activator like effector nucleases (TALENs) [82]. Although the CRISPR/Cas system has recently received much attention due to broad use in genome engineering of patient cells [83], ZFNs are also popular for treating graft-versus-host disease in T-cell therapy [84].

Recently, a nuclease dead variant of Cas9 bearing a transcriptional trans-activator has recently been used in cellular reprogramming by activating the transcription factors Oct4 and Sox2, which maintained pluripotency and expressed the markers for the three germ layers [85].

Although genetic modification is a rate-limiting step in the cell manufacturing industry, the conventional methods make it more complicated because it is a multi-step process. Conventional genetic modification in planar culture is also costly, labor-intensive, and time-consuming. The bioreactor is a better platform for producing large-scale genetically modified cells for commercial purposes because cell expansion is possible in the same vessel which makes the process straightforward (**Figure 2**). For genetic modification in bioreactor, Hsu et al. recently reported how to transfect reprogramming factors in bioreactor where they tried eliminating viral vectors for gene delivery by using cationic reagents [78]. Generally, transfection of reprogramming factors for generating induced pluripotent stem cells (iPSCs) is done in adherent culture and then cells are expanded in 2D or 3D which is a two-step process. By integrating the genetic modification step in bioreactor, it is possible to establish a single-step process which enables cell manufacturing in automated and closed bioreactor system.

Genetic modification is also a challenging step in CAR-T cell therapy-based products. In CAR-T cell therapy, generally cells are isolated from patients' blood sample and then the cells are expanded after selection and activation. Finally, the cells need to be transduced with the CAR or any other antigens depending on target diseases. Conventional methods for genetic transduction are based on planar culture where every step is performed in open culture system. Recently, a few steps are integrated in bag culture system where selection, activation, and expansion can be done in a single step using DynaMag™ CTS™ [86], whereas the Xuri cell expansion System developed by GE Healthcare can expand cells in large numbers [87–89].

Although washing and concentrating the final product are integrated by the COBE® 2991 system developed by Terumo BCT [90], the transduction step is still not integrated in any of the above systems. Integrating the transduction step with the expansion and formulation will make the CAR-T cell therapy straightforward and performing these steps in bioreactor is a good platform since the physiological parameters as well as automated operation is possible in bioreactor culture. Miltenyi Biotec developed a device named CliniMACS Prodigy™ which is based on bag culture for CAR-T cell therapy. This device integrated major steps especially cell preparation, selection, activation, expansion, transduction, washing, and formulation in an automated system [91–93]. Such integration in the bioreactor will pave a straightforward method for producing cell-based products in a closed and automatic method.

3.2 Integrated system for large-scale expansion and differentiation in bioreactor

Current manufacturing practices for stem cell-based products are multi-step: derivation, expansion, and differentiation. In this process, patient-derived skin fibroblast cells are transduced with reprogramming factors in the planar culture. After deriving hiPSCs, cells are expanded in planar or bioreactor culture to obtain a large number of cells. Then cells are differentiated to target cells of interest. The differentiated cells are characterized and transplanted to the patient in a dose-dependent manner. As this process is complicated with multiple steps, it poses high risk of contamination to the final products. Moreover, maintaining cGMP culture

platform is also mandatory for cell-therapy products [94–96], which makes the cell manufacturing process more complicated. Therefore, developing an integrated system that can combine all these steps from derivation to final products is required. Here, the bioreactor may be a good platform for doing this (**Figure 2**).

The bioreactor platform is widely used for the large-scale expansion of hPSC-based cell therapy production because bioreactor is easy to operate in an automated mode where various physicochemical parameters can be regulated in a closed-system. Two groups have demonstrated that the bioreactor is conducive to cellular reprogramming [97, 98]. Shafa et al. reported a significantly higher reprogramming efficiency in the bioreactor compared to the planar culture [97]. Since mesenchymal-epithelial transition (MET) is an important early step in cellular reprogramming [99], transformed fibroblasts that are moved into the bioreactor will form aggregates that are efficiently expanded in the bioreactor. Indeed because fibroblasts are substrate-dependent, bioreactor culture may be promoting aggregate formation and therefore cellular reprogramming.

Unfortunately, bioreactor reprogramming methods require genetic modification (retroviral, piggyBAC) prior to bioreactor expansion. It is theoretically possible to pursue cellular reprograming fully and completely in the bioreactor. Recently, for example, Hsu et al. has demonstrated that it is possible to transfect human fibroblasts directly on microcarriers [70]. Reprogrammed cells in theory will leave the microcarrier to form aggregates in the bioreactor via MET.

Following bioreactor derivation of hPSCs, the next big steps are expansion and differentiation. Generally, a large number of cells are required for an effective cell therapy application, which is ranging from 10^8 to 10^{10} cells per 70 kg patient [100]. In the conventional process, cell expansion is performed in planar culture. However, it has many drawbacks and limits the cell expansion in various ways. Planar culture is unable to provide enough growth surfaces for the unlimited expansion.

Another major drawback is surface coating. Extracellular matrix (ECM) is needed for surface coating which is initially derived from animal sources, which poses high risk in clinical-grade manufacturing. Currently, recombinant ECM has been discovered, which can be used efficiently for clinical applications [101]. The advancement in cell coating also stimulated the advancement of integration and automation of cell expansion in adherent culture.

Automated planar culture systems have been established for the expansion of hPSCs for clinical-grade cell manufacturing. One of the notable automated systems for cell manufacturing is CompacT SelecT™ developed by the TAP Biosystems. This system is based on T-flask where 90 T175 flasks can be accommodated for large-scale expansion of cells. All the cell culture steps, cell counting, seeding, medium change, passaging, and plating as well as transient transfection can be done automatically by using this robotic system. However, such systems are not used for differentiation since differentiation is a complicated process, which needs several components to add in the culture medium. As a result, the expansion and differentiation process in planar culture is mostly disintegrated.

Cell expansion in bioreactors need not require surface coating except for microcarrier culture. Bioreactor also provides enough growth surface availability. Generally, a single bioreactor (100 mL working volume) is enough for providing clinically relevant number of cells for autologous cell therapy applications. Several types of bioreactors are employed for the expansion of hPSCs [102]. For anchorage-dependent expansion of hPSCs, microcarriers need to be coated with ECM for cell attachment in the bioreactor [100, 103–105].

After large-scale expansion, cells are harvested by detaching them from the microcarrier using enzymatic treatment. Nonenzymatic detachment is also available by changing temperature or pH [18–20]. Bioreactor expansion of hPSCs on

microcarrier is troublesome for clinical application because it needs an extra step for microcarrier separation from the final cell harvest. On the other hand, aggregate culture in bioreactors may not necessarily need a detachment step for harvesting [21–25] and clinically relevant numbers of cells can be produced in a single bioreactor as aggregate [21, 106–108].

A major drawback in aggregate culture is the size limitation. With the increase in aggregate size, the growth potential decreases in the large size aggregate due to diffusion limitation of oxygen and nutrients [109]. Therefore, maintaining aggregate size is an important issue to maintain high growth rate as well as high quality for cell therapy applications [21].

After expansion, cells can be differentiated in the same vessel which makes bioreactor culture a unique choice for integrated biologics manufacturing. Bioreactors were used for differentiation of hPSCs into various cell types, especially for cardiac [110–112], hepatic [113, 114], and neural [115] lineages. To provide straightforward methods for clinical applications, integration of expansion and differentiation is important and there are several reports published recently where expansion and differentiation were integrated [108, 116–118]. However, the integration of derivation with expansion and differentiation is still facing complications and there are a very few reports available.

Steiner et al. reported integration of derivation, propagation and differentiation of hESCs in suspension culture where hESCs were isolated from the inner cell mass in suspension culture that did not involve feeder cells or microcarriers [119]. However, the integration of derivation, expansion, and differentiation is not still realized for personalized medicine especially for autologous or allogenic cell therapy applications. Such integration is needed for overcoming the multi-step cell processing, which will reduce the risk of contamination and save cell processing time as well as reduce manufacturing costs for cell therapy manufacturing.

4. Concluding remarks and future directions

Cell therapy applications utilizing stem cells are increasing day by day and several clinical trials are ongoing to treat incurable diseases. With the growing need for cell-based products, the manufacturing facilities should be compatible for fulfilling the market demand by supplying safe and effective cell-based products. Since the current manufacturing systems are stuck with several drawbacks, especially multi-step processing which poses high risk of contamination as well as long processing time which contributes to increase culture cost, a more straightforward system is required. Bioreactor-based cell manufacturing system can provide a single-step and straightforward processing of cell-based products. Integration of different steps, especially genetic modifications, derivation, and expansion as well as differentiation in bioreactor will pave the future of manufacturing cell-based products. The integrated biologics manufacturing in stirred suspension culture will significantly reduce the risk of contamination of final products, increase product efficacy, and reduce cell processing time and provide a cost-effective platform for cell manufacturing for cell therapy applications.

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SCN conceptualized, designed, and wrote the manuscript. DER conceptualized and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

A Simple Way to Produce Gold Nanoshells for Cancer Therapy

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Abstract

Gold nanoshells (GNSs), formed by a silica core surrounded by a gold shell, present a shift on their surface plasmon resonance (SPR) to the near-infrared (NIR) part of the electromagnetic spectrum when synthesized with specific dimensions. This chapter presents a simple method to prepare the nanoshells, a step-by-step characterization, as well as their absorbance spectrum. For the synthesis, silica spheres, with approximately 190 ± 5 nm in diameter, were prepared using the Stöber method and then functionalized with 3-aminopropyltriethoxysilane (APTES). The gold nanoparticles (GNPs), with a diameter of 7 ± 3 nm, were produced by the reduction of chloroauric acid. Then, the silica was seeded with the GNPs to later grow a gold shell with the help of $Au(OH)_4^-$ ions and formaldehyde. UV-Vis spectroscopy results showed an increase of absorbance starting at 520 nm. It reached its maximum around 600 nm and kept absorbing all through 1200 nm. Transmission electron microscope (TEM) and scanning electron microscope (SEM) images suggest that the absorption peak movement coincided with the completion of the shell. Furthermore, when the sample was irradiated with an 820 nm wavelength/3.1 mW laser, its temperatures increased by 6.3°C in 2 min, showing its absorbance in the NIR.

Keywords: gold, nanoshells, surface plasmon resonance, near-infrared absorption, silica core, core-shell particles

1. Introduction

There are hundreds of types of cancer, and each one has different characteristics [1]. Therefore, science utilizes the most innovative discoveries in an effort to find new treatments, and nanotechnology offers a wide variety of options. One example of this is the nanoparticle colloids. They can be designed to concentrate on specific organs (passive targeting), or their surfaces can be modified by an antibody or ligand to get attached to a specific target (active targeting) [2]. Furthermore, metallic nanoparticles, like GNS, present interesting optical properties. The shell, formed by GNPs, confines the plasmons to the surface of the particle, changing the plasmon frequency of the gold. Therefore, the GNSs absorb different wavelengths than gold

in bulk. Moreover, when the wavelength of the incident light is larger than the size of the nanoparticle exciting the plasmons at their natural resonance frequency, light is absorbed more strongly causing an increase in temperature. When the GNSs are synthesized with specific geometry and dimensions, their SPR changes causing their absorption to shift to the NIR region of the electromagnetic spectrum [3]. This shift offers a great potential for applications in the medical field because GNPs are bio-inert [4], and the cytotoxicity of the silica has been widely studied [5]. Additionally, the wavelengths of the NIR spectrum are considered the optical window of the human body. As a result, while most biological soft tissues have low absorption of these wavelengths [6], GNSs absorb them causing them to increase their temperature.

GNSs have been synthesized over different templates. Polystyrene cores claim to offer a narrower plasmon resonance absorption peak due to their higher reflective index [7]; iron oxide nanoparticles present a superparamagnetic template useful for magnetic resonance imaging [8]; silver nanoparticles have also been used as a mold for hollow gold nanoshells [9]. However, the functionalization of the polystyrene takes more time, reactants, and supervision which increases the chances of error as compared with the functionalization of the silica. Besides, the cytotoxicity of the silica nanoparticles makes them a good option for medical applications. Moreover, once the GNS is produced, the silica core can be diluted with hydrochloric acid to obtain hollow gold nanoparticles [10] that can be used for the controlled release of drugs [11] due to their capacity for encapsulating sensitive materials and their low thermal expansion coefficient. Therefore, providing a simpler and more efficient method of synthesis of GNS on silica templates provides a more promising variety of applications like for photothermal therapy [12], optical imaging [13], and drug release [2], as well as providing a near instantaneous in situ whole blood assay [14].

The synthesis of the GNSs has been extensively explored. Different methods, like reflux systems [15] or flow micro-reactors [16], can be used as well as procedures involving high temperatures [17]. But most of those methods last over 30 h [18]. In this chapter, we present a simple and effective method of preparation that shortens the time of the traditional procedures published before and uses only a magnetic stirrer with heating for the synthesis.

The reductions of the time were obtained by first modifying the Stöber method of synthesis of silica particles from 2 h to 30 min Samples were obtained at 30, 60, 90, and 120 min throughout the reaction to determine the minimum time of reaction needed. Also, the seeding process can be shortened from 2 h to 30 min. During the seeding process, where the silica is decorated with GNPs, a sample was obtained using only 30 min of resting time and compared with another sample obtained after the full 2 h of the resting time previously suggested. In both cases, SEM images were obtained showing that 30 min were sufficient to accomplish the synthesis of the silica as well as their seeding. In consequence, the total time of the process was reduced by 3 h.

2. Background

The "Birth of Nanotechnology" was the title used by David Thompson [19] on his article acknowledging Michael Faraday's synthesis of gold nanoparticles in 1857. What Faraday called "Colloidal Ruby Gold" [20] was, in fact, a solution of dispersed GNPs so small that no microscope of that time was able to observe them. It wasn't until 1985 that Turkevich et al. [21] used an electron microscope to corroborate that Faraday's ruby gold was formed by GNPs with an average size of 6 ± 2 nm. Separately, in 1967 Werner Stöber et al. developed a method of synthesizing silica spheres in the micron size range [22] to be used especially in the medical field due to its known cytotoxicity, and in 1998 they were used by Naomi Halas et al. as the templates of GNS [23].

3. Experimental

3.1 Materials

Ethanol (100%), tetraethyl orthosilicate (TEOS) (98%), 3-aminopropyltriethoxysilane (APTES) (99%), trisodium citrate dihydrate, gold (III) chloride trihydrate ($HAuCl_4$, 49%), formaldehyde (37%), and sodium borohydride ($NaBH_4$, 98%) were purchased from Sigma-Aldrich. Potassium carbonate (K_2CO_3, 99%) and ammonium hydroxide (28%) were purchased from J.T. Baker. All the solutions were prepared with deionized water.

3.2 Characterization

Images were obtained using the field-emission scanning electron microscope (SEM, JEOL JSM-7401F) and the transmission electron microscope (TEM, HT7700 Hitachi). For the ultraviolet-visible (UV-Vis) spectra, the Evolution 220 spectrophotometer UV-Vis (Thermo Scientific) was used. The FTIR spectra were obtained with an IRAffinity-1S Fourier transform infrared spectrophotometer (Shimadzu). The sample was irradiated with an 820 nm wavelength/3.1 mW laser (Multi-Channel Fiber-Coupled Laser Source, Thorlabs), and the infrared images were taken with a Non-contact Digital IR Thermometer (TrueIR Agilent Keysight U5855A). Measurement of the particles and histograms were acquired with the Image J® software [24].

3.3 Preparation of silica spheres

Silica particles were prepared by modifying the Stöber method [22]. About 50 ml of ethanol, 2.5 ml of deionized water, and 4.25 ml of ammonium hydroxide were magnetically stirred in an 80 ml glass flask for 5 min. Then, 0.75 ml of TEOS was added dropwise. The solution was heated at 40°C. Temperature and agitation were kept for 2 h. The color of the solution changed from transparent to opaque white approximately 10 min after adding the TEOS as shown in **Figure 1**. This time corresponds to the induction period needed to form the SiO_2 nucleus from the concentration used of the TEOS monomer [25]. Samples were obtained at 30, 60, 90, and 120 min after adding the TEOS to observe the evolution of the process.

3.4 Functionalization of the silica spheres

In order to create open links over the silica to attach the GNPs, the silica was functionalized with APTES on a 1 ml:1 μl silica/APTES volume ratio. About 50 ml

Figure 1.
Images of the synthesis of SiO_2 particles: (a) right after adding TEOS, (b) at 10 min of reaction, (c) at 30 min of reaction, and (d) at 2 h of reaction.

of the silica template solution was magnetically stirred for 5 min with 50 µl of APTES in an 80 ml glass flask. The solution was left still overnight at room temperature. The opaque white functionalized silica particles precipitated in the solution leaving a clear fluid at the top. To separate the functionalized silica, the mixture was centrifuged at 6000 rpm for 1.5 min and washed in deionized water three times. Finally, they were sonicated in 20 ml of deionized water final volume.

3.5 Synthesis of gold nanoparticles

The method presented by Abdollahi et al. [10] was followed to elaborate the GNPs. First, 100 ml of deionized water at room temperature was placed in a 140 ml flask under magnetic agitation. Then 1 ml of 1% $HAuCl_4$ solution, 2 ml of 1% trisodium citrate, and 1 ml of freshly made 0.075% $NaBH_4$ in 1% trisodium citrate were added in that order. The mixture was stirred for 10 min and used immediately to avoid the agglomeration. The GNP may also be stored at 4°C in an amber glass bottle for later use.

Throughout the synthesis, the gold solution changed its color from light yellow (**Figure 2a**) to wine red (**Figure 2b**). This is a characteristic of the GNP formation [26].

3.6 Seeding process

For the seeding process, 100 ml of GNPs and 10 ml of functionalized silica were magnetically stirred in a 140 ml glass flask for 5 min as shown in **Figure 3a**. Then, it was left still for 2 h. **Figure 3b** presents how the seeded silica spheres precipitated and changed their color from opaque white to lavender, while the mother solution changed from wine red to transparent. The mixture was centrifuged at 6000 rpm for 2 min and washed in deionized water three times. Finally, it was sonicated in 20 ml of deionized water final volume. The same procedure was followed, but the solution was left still for only 30 min to observe the development of the seeding process through time.

3.7 Gold hydroxide solution

For the shell growth process, a gold hydroxide solution was prepared by mixing 100 ml of 2 mM K_2CO_3 solution and 1.5 ml of 1% $HAuCl_4$ in a 140 ml glass flask for

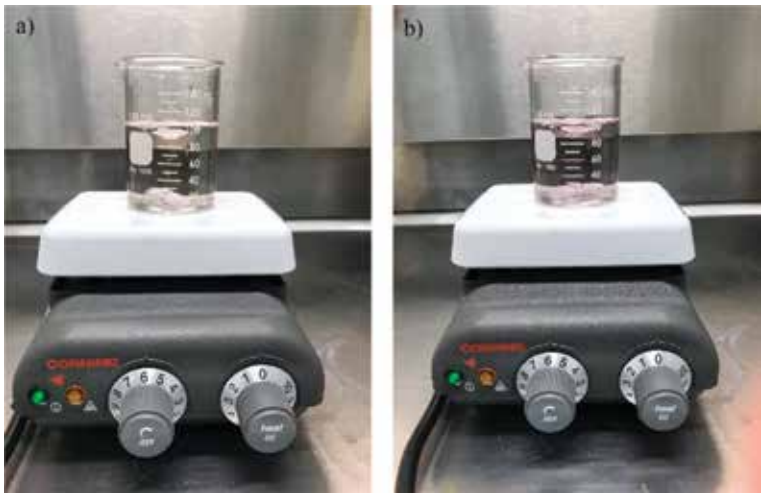

Figure 2.
Synthesis of GNPs at (a) the beginning of the reaction and (b) after 10 min of reaction.

Figure 3.
Images at (a) the beginning of the seeding process and (b) after 2 h of resting time.

30 min. The color of the solution changed from light yellow (**Figure 4a**) to transparent (**Figure 4b**). It was left still overnight at room temperature in an amber glass bottle to facilitate the formation of $Au(OH)_4^-$ ions [18].

3.8 Shell growth

The shell was developed from the gold seeds deposited over the functionalized silica particles with the help of the $Au(OH)_4^-$ ions. About 100 ml of the gold hydroxide solution (**Figure 5a**) and 5 ml of seeded silica were magnetically stirred in a 140 ml glass flask for 5 min (**Figure 5b**). Next, 5 ml of formaldehyde was added to the solution (**Figure 5c**) and stirred for 10 min (**Figure 5d**). The solution was left still for 50 min. Finally, it was centrifuged at 6000 rpm for 2 min, washed, and dispersed in 10 ml of deionized water final volume.

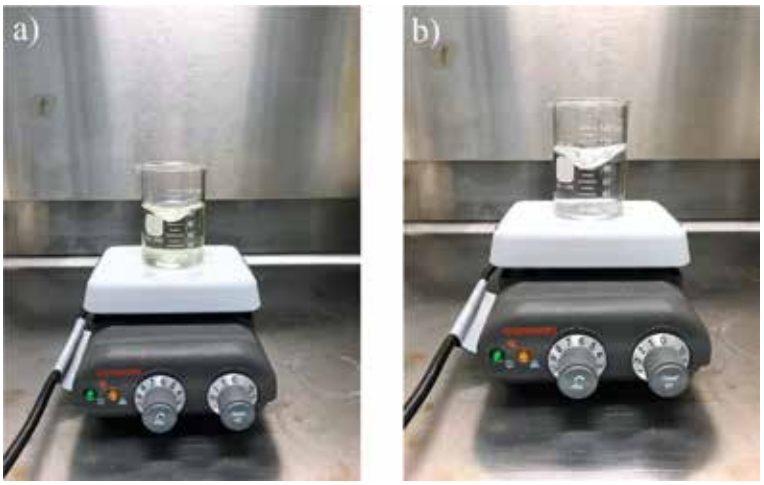

Figure 4.
Images illustrating the change of color of the gold hydroxide solution at (a) the beginning of the synthesis (light yellow) and (b) 30 min of reaction (transparent).

Figure 5.
Images of the shell growing process. (a) Gold hydroxide solution, (b) gold hydroxide + seeded silica, (c) gold hydroxide + seeded silica + formaldehyde, and (d) solution after 10 min of reaction.

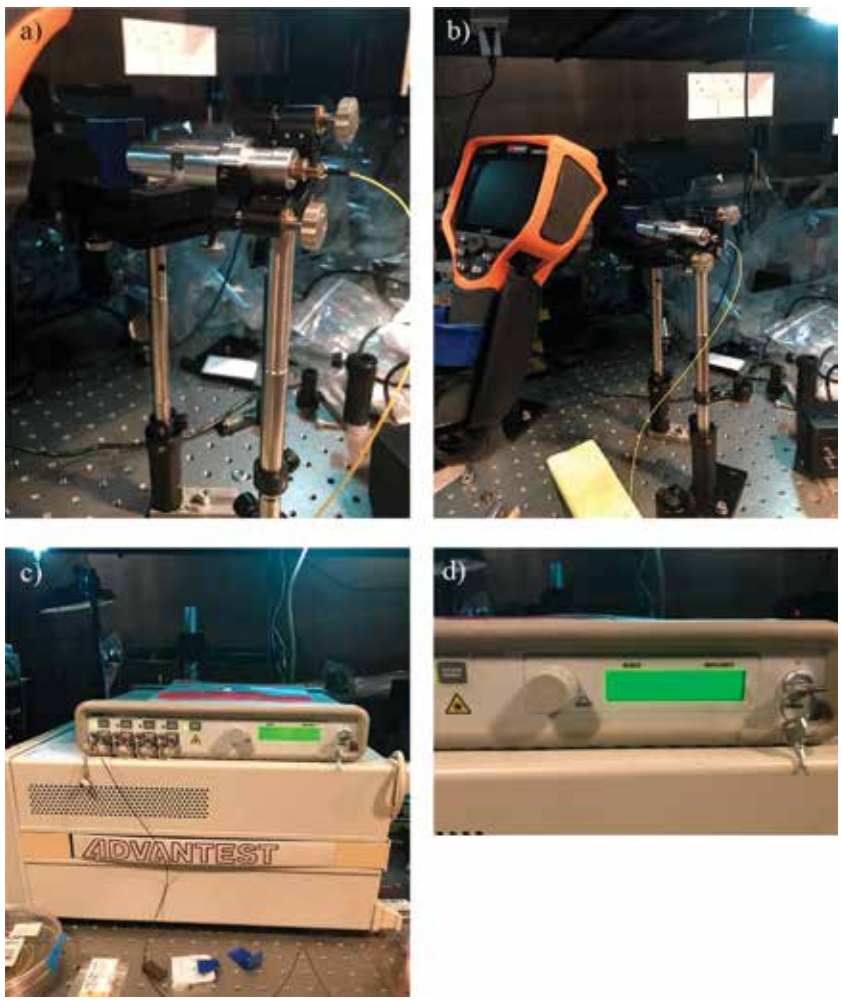

Figure 6.
Images of the installation of the (a) 820 nm wavelength laser, (b) non-contact digital IR thermometer, (c) connection to the multi-channel laser source, and (d) selection of the channel with the desired wavelength.

3.9 Thermography

To obtain the IR images, first, the 820 nm wavelength laser was fastened to the support for it to aim directly to the sample (**Figure 6a**). Then the Digital IR

thermometer was also secured and directed to the GNS (**Figure 6b**). Next, the laser was connected to the Multi-channel laser source (**Figure 6c**). Finally, the channel with the desired wavelength was selected (**Figure 6d**), and the irradiation was started.

4. Results and discussion

4.1 Characterization of the silica templates

TEM images obtained from the silica samples taken at 30, 60, 90, and 120 min after adding the TEOS are presented in **Figure 7**. When comparing the images, no significant variation in the size of the silica particles is noticeable.

To corroborate that the silica particles do not change substantially when the reaction time is over 30 min, the images were studied with the software Image J®, and the diameter distribution of the particles was analyzed. Over 1000 particles from the different samples were measured to obtain the histograms presented in **Figure 8** where samples A, B, C, and D correspond to 30, 60, 90, and 120 min of reaction time, respectively. They illustrate that the diameter distribution of the silica spheres throughout the synthesis oscillates around the 190 ± 5 nm on all the samples.

To have a better understanding of the information, **Table 1** contains useful statistic information from the samples.

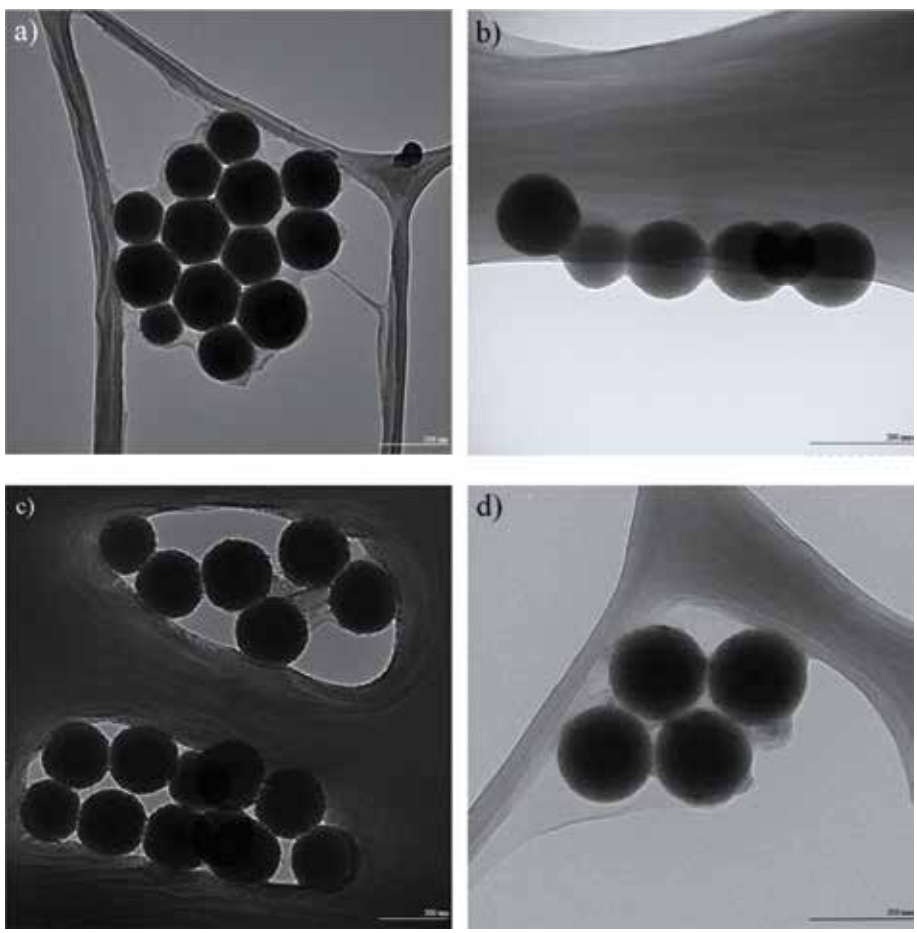

Figure 7.
TEM images of silica particles at (a) 30, (b) 60, (c) 90, and (d) 120 min after adding TEOS.

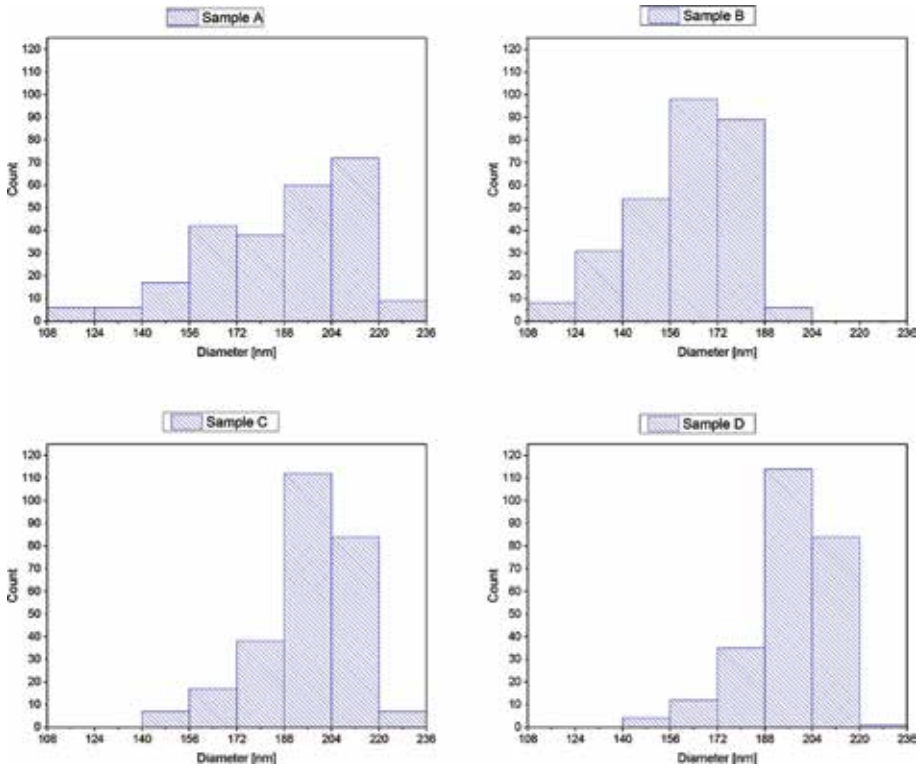

Figure 8.
Histograms illustrating the diameter distribution of the silica particles throughout their synthesis.

	Count	Mean	Std dev.	Mode
Sample A	250	187	25	197 (79)
Sample B	287	162	18	162 (80)
Sample C	265	196	16	201 (92)
Sample D	250	197	13	198 (105)

Table 1.
Statistic information obtained by measuring the diameters of silica particles from the different samples.

The mean, standard deviation, and mode obtained after analyzing the samples show that, in general, the silica templates keep their size and shape after 30 min of synthesis. Therefore, the objective of synthesizing silica particles with diameters of 190 ± 5 nm was achieved within 30 min of reaction time. More than 30 min of synthesis does not result in any relevant change in the sample. For this reason, the total process time can be reduced from 2 h to 30 min, shortening the reaction time by 1 h and 30 min when compared with similar published works where the synthesis time is at least 2 h [10, 17, 27, 28].

4.2 Characterization of the functionalized silica templates

The functionalization of the silica with a primary amine group ($–NH_2$) was accomplished by the use of APTES which changed the superficial charge of the

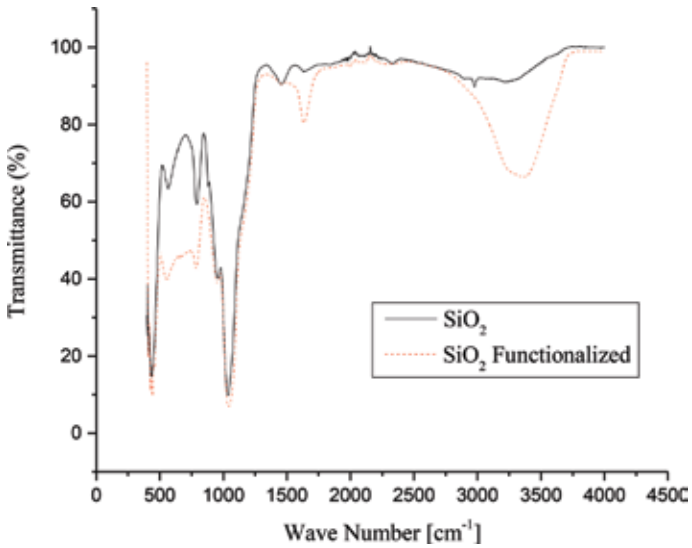

Figure 9.
FTIR spectrum of silica particles and silica particles functionalized with APTES.

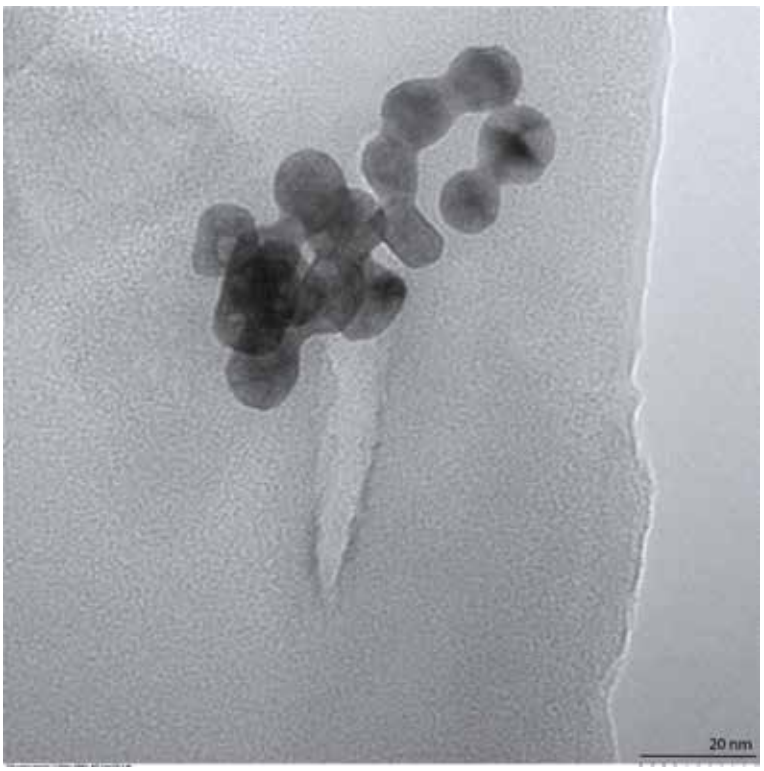

Figure 10.
TEM images of GNPs.

silica providing an electrostatic link for the GNPs to attach [29]. This superficial modification was verified by the FTIR spectrum shown in **Figure 9** where the vibrations of primary amines are found between 3550 and 3330 cm^{-1} which correspond to the vibrations of a primary amine group [30].

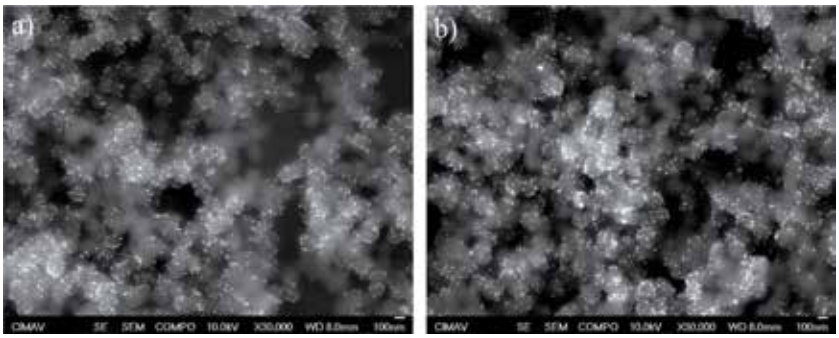

Figure 11.
SEM images illustrating the seeding process with (a) 30 min of resting time and (b) 2 h of resting time.

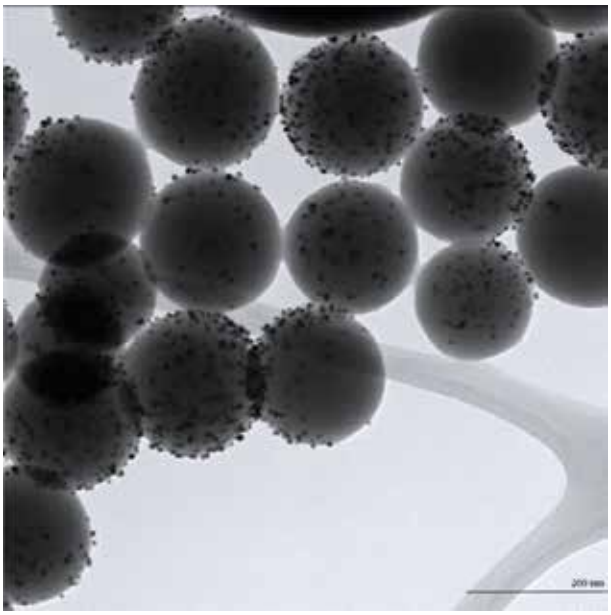

Figure 12.
TEM image of a gold decorated silica particle.

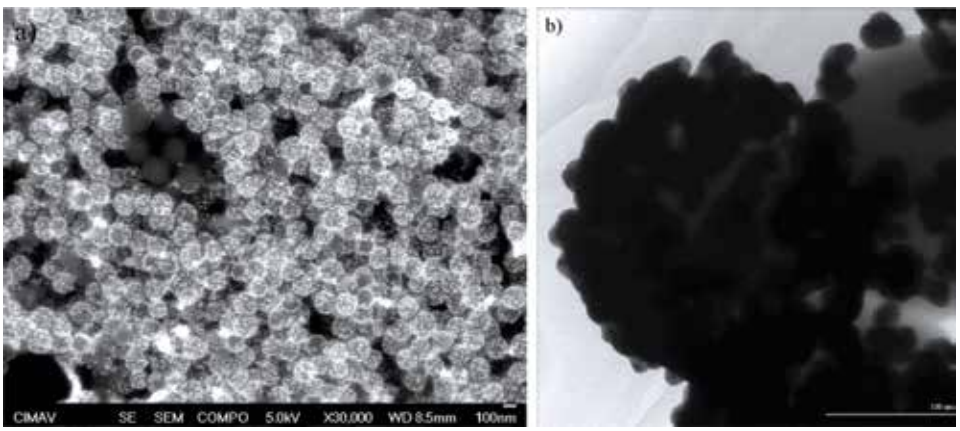

Figure 13.
(a) SEM and (b) TEM images of GNS.

4.3 Characterization of the gold nanoparticles and seeded silica

The GNPs were analyzed under a TEM. **Figure 10** illustrates the GNPs with a diameter of 7 ± 3 nm and spherical shape overall.

The seeding process was followed with 2 h of still time as well as with 30 min of still time. The first and second samples were observed under the microscope. The samples were taken with the purpose of observing the development of the seeds. **Figure 11a** presents an SEM image of seeded silica with 30 min of resting time, while **Figure 11b** presents an SEM image of seeded silica with 2 h of resting time. The images show that 30 min is enough time to create the seeds because both images display approximately the same number of nucleus per silica particle.

Even though a complete shell was not formed, the seeds are ready to grow the gold shell on the next step. A TEM image of a seeded silica particle is presented in **Figure 12**. This image corroborates the seeding process as well as the silica functionalization.

4.4 Characterization of the gold nanoshells

Figure 13a and **b** presents SEM and TEM images of the synthesized GNS, respectably. They illustrate that the silica particles are almost surrounded by gold. The higher density of gold, the separation of the GNPs [31], and the dielectric properties of the silica [3] contribute to the absorption of the NIR wavelength, which causes the increase in temperature.

4.5 The UV-Vis spectrum

Figure 14 presents the UV-Vis spectrum of the particles through the process. Silica particles, as well as functionalized silica particles, do not show significant

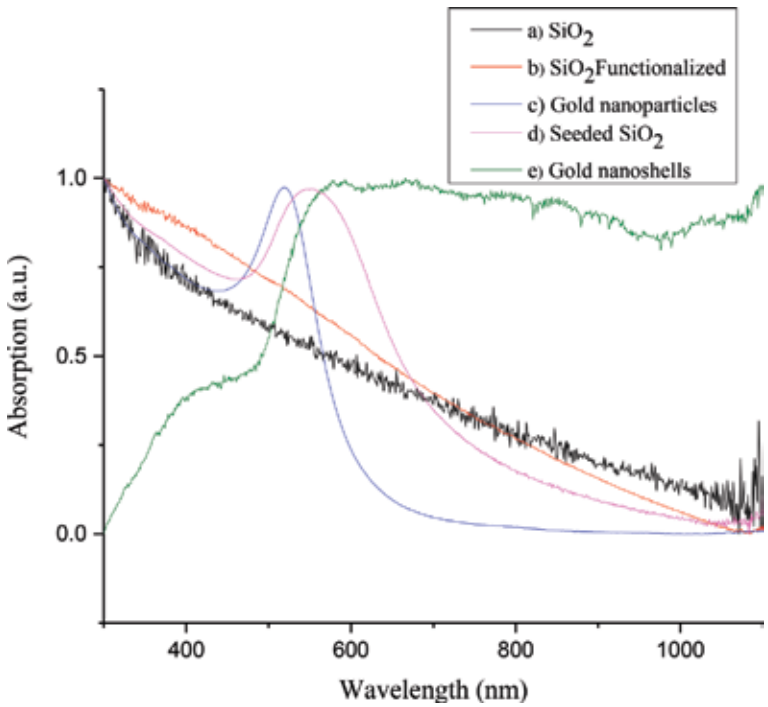

Figure 14.
UV-Vis spectrum of (a) silica, (b) functionalized silica, (c) gold nanoparticles, (d) gold seeded silica, and (e) gold nanoshells.

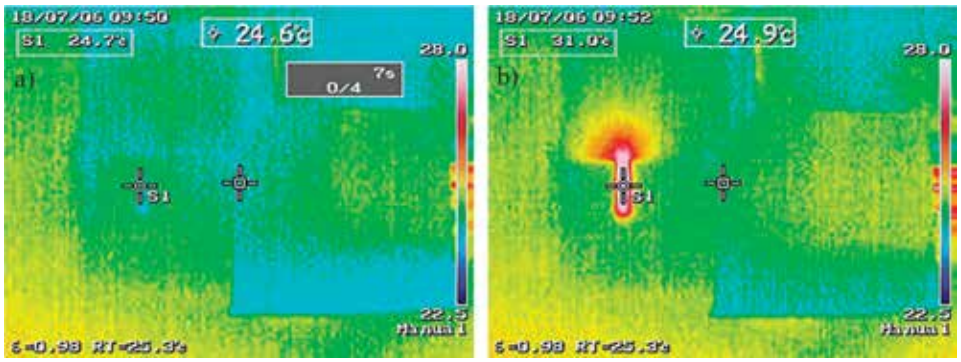

Figure 15.
Thermography images of the GNS taken (a) before and (b) after being irradiated with 840 nm wavelength laser for 2 min.

absorption on the NIR. As for the GNPs, they exhibit their characteristic absorption between 520 and 530 nm [29]. However, on the seeded silica particles, the slight shift to the NIR is noticeable. While on the GNS, the peak not only shifted to the NIR, but it kept a high absorbance all the way to 1100 nm. This range is part of the optical window of the human body [5]. The absorbance of the GNSs is due to the SPR that creates an electric field on the surface increasing the absorption of these wavelengths. SPR happens when metal nanoparticles are irradiated with a wavelength bigger than their size, exciting the electrons of the conducting band [2].

4.6 Heat generation of GNS from light energy

To verify the absorbance of the GNS, they were irradiated with an 840 nm laser with a power of 3.1 mW. **Figure 15a** and **b** displays the thermography images of the sample while being irradiated at time zero and 2 min later. The temperature of the sample increased from 24.7 to 31.0°C. This confirms that GNSs are able to absorb light from the NIR and convert it in heat.

5. Conclusions

Synthesizing GNS by seeding and growing a gold shell over silica spheres with GNPs showed to be an effective method to tune their absorption to the NIR. The SEM and TEM images show the evolution of the process, while the absorbance spectrum displays the GNS shifting over the NIR. Therefore, we obtained a simple technique of producing GNS that can be used for medical applications thanks to the bio-inert GNPs [3] and the widely studied cytotoxicity of the silica [5]. This method does not require long periods of time, when compared with previously published mechanisms, and does not need sophisticated equipment.

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Nomenclature

GNSs	gold nanoshells
SPR	surface plasmon resonance
NIR	near-infrared
APTES	3-aminopropyltriethoxysilane
GNP	gold nanoparticles
TEM	transmission electron microscope
SEM	scanning electron microscope
TEOS	tetraethyl orthosilicate

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Chapter 7

Engineering of Surface Proteins in Extracellular Vesicles for Tissue-Specific Targeting

Stefan Vogt, Gerhard Stadlmayr, Johannes Grillari, Florian Rüker and Gordana Wozniak-Knopp

Abstract

Extracellular vesicles (EVs) have in the recent decades gained an important stand as vehicles enabling cell-to-cell transport and communication. With the advanced development towards their clinical use and increasing versatility of potential applications, improving their tissue-specific targeting in order to enhance their functionality in drug delivery opened as a challenging engineering field. In the past, the question of specific intercellular contact has been addressed by decoration of the EV surface with agents able of specific target recognition. An attractive possibility here is the modification of strongly overexpressed EV surface marker proteins towards recognition of target cells. As these proteins are involved in a plethora of biological functions in EV biogenesis, cargo targeting and intercellular transfer, a minimal impact on protein architecture upon modifications is desirable, which would also increase the stability of the exosomal preparation intended for therapeutic use. This chapter focuses on the possibilities of engineering of the EV marker proteins towards antigen-recognition units broadly applicable to endow EVs with tissue-targeting functionality.

Keywords: extracellular vesicles, exosomes, tetraspanin, tissue-specific targeting, exosomal drug delivery

1. Introduction

The transfer of extracellular vesicles (EVs) has emerged in the last two decades as a novel mechanism for intercellular communication. Nomenclature of EVs has by now been agreed to be based on biogenesis pathway. Therefore, EVs budding from plasma membranes are termed ectosomes or microvesicles, while exosomes are formed via the endosomal compartment within multivesicular bodies (MVB) which then are released by their fusion with the plasma membrane. Finally, when blebbing from apoptotic cells, the term apoptotic bodies has been retained [1]. Biomarkers to differentiate these EVs are still questionable, and in general, size is used to differentiate exosomes from ectosomes, while apoptotic bodies are considered to present phosphatidylserine on the outside and thus can be stained by annexin V. Exosomes (40–150 nm in diameter) are produced by formation of endosomal intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are secreted by fusion of these vesicles with the plasma membrane [2, 3] (**Figure 1**).

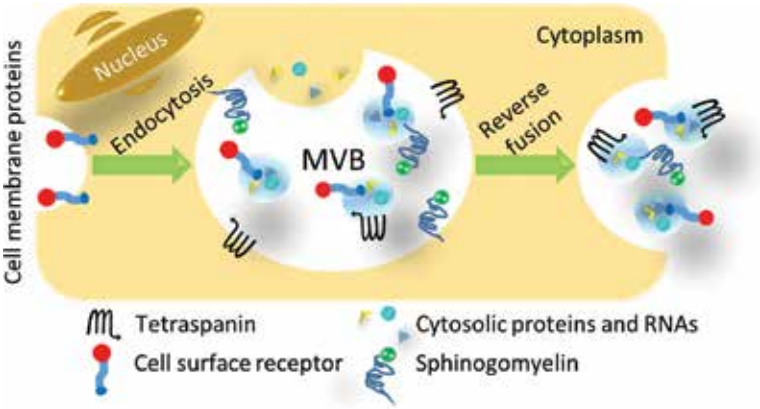

Figure 1.
Biogenesis of EVs.

Since they have been known to mediate directional intercellular transfer of their content, typically proteins, mRNAs, microRNAs (miRNAs) and a variety of non-coding RNAs [4], several studies were oriented towards their application as drugs *per se* or as therapeutic tool for drug delivery [5]. Their membrane is strongly enriched in sphingomyelin and cholesterol, which contributes to their unique buoyancy coefficient and enables practical isolation from other particles of cellular origin using differential centrifugation methods. Another discernible feature of exosome membranes is that it reflects the composition of the MVB membrane and has a high density of endosomal membrane proteins, such as proteins involved in MVB biogenesis (Alix and TSG101), membrane transport, in particular the components of the endosomal sorting complex required for transport (ESCRT) and most prominently tetraspanin proteins (CD9, CD37, CD53, CD63, CD81, CD82 and CD151) [6, 7]. Some tetraspanins such as CD9, CD81 and CD151 are more broadly expressed, while others are restricted to specific EV subsets [8, 9]. The overexpression of tetraspanins, as shown for CD9, can in several production cell lines enhance the exosome production and, in addition, cause a reduced overall size of the vesicles [10].

Tetraspanins play an important role in several physiological processes [11] and have been discovered to cooperate in states of health and disease in signal transduction, cellular activation, polarization, motility, adhesion, tissue differentiation, angiogenesis, tumorigenesis and metastasis [12–14], both by regulating cellular interactions as cell-membrane bound molecules and indirectly through exosomes. They are involved in each step of the metastatic cascade due to their ability to interact with cell surface receptors, adhesion molecules, matrix-remodeling proteases and signaling molecules. In this pathological state, they hence regulate cell proliferation, participate in epithelial-mesenchymal transition, modulate integrin-mediated cell adhesion and mediate the invasion through modulation of angiogenesis, tumor-endothelial cell interactions and regulation of cancer cell migration through the regulation of tumor microenvironment, as well as direct influence on extracellular matrix [15]. In this chapter, we introduce the characteristics of EVs and the engineering approaches aimed at their surface proteins to achieve tissue-specific targeting.

2. Structure of the tetraspanin proteins

The rod-shaped structure of a tetraspanin consists of four transmembrane helices that connect the two extracellular loops [16] (**Figure 2**). The short extracellular loop (SEL), which has not yet been reported to contain any element of a

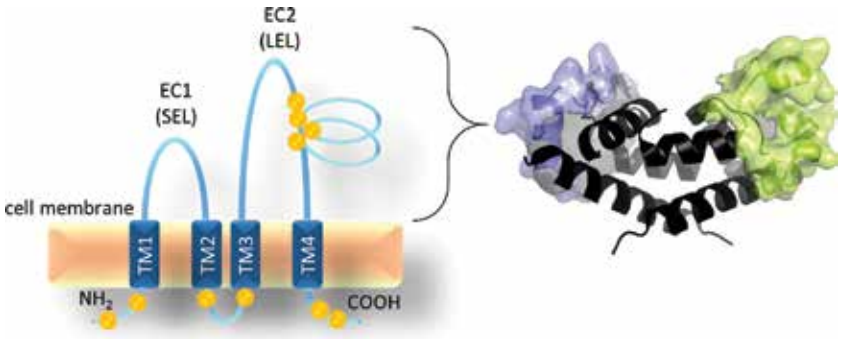

Figure 2.
Organization of tetraspanin CD81: 4 transmembrane helices (TM) span 2 extracellular loops. Positions of cysteine residues are indicated with yellow dots. Crystal structure of EC2 domain is presented as a cartoon diagram of the dimeric form (PDB: 1G8Q); the conserved three-helix bundle is black and the variable domains C and D are surfaced in blue or green for each protomer. The figure was prepared using PyMOL (PyMOL molecular graphics system, version 1.3 Schrödinger, LLC).

particular secondary structure, is of 12–31 amino acids in length. In the studies oriented towards tetraspanin engineering, the EC1 domain has until now been less addressed due to its lower degree of structural organization. Its replacement for a strand of glycine-serine residues has led to an unusual cell surface expression and a distribution dissimilar to the wild-type parental molecule, which may suggest the importance of this structural subunit for the stability of the membrane-bound tetraspanin [17].

The large extracellular loop (LEL) or extracellular domain 2 (EC2) has on the other hand been well characterized. The crystal structure of CD81 [18] demonstrated the four invariant cysteine resides within EC2 to form the 2 disulfide bridges, as a hallmark stability feature of the conserved tetraspanin fold [8, 19] (**Figure 2**). Moreover, the topology of several other tetraspanins was predicted by molecular modeling studies in the absence of an available crystal structure, using the CD81 EC2 structure as a template: these were the tetraspanins CD37, CD53, CD82 and CD151 [20, 21]. It has been early established that binding of CD81-specific antibodies depends on the formation of the disulfide bonds [22]. One class of tetraspanins harbors the EC2 composed of five α-helical elements, forming stalk and head elements of a mushroom-like structure, and the second class of tetraspanins is postulated to contain an additional helical element, stabilized with another pair of cysteine residues. The EC2 fold structure is evolutionarily conserved across species for any particular tetraspanin in spite of a high degree of variability at the amino acid level [23], with a subdomain consisting of a three-helix-bundle fold and a second subdomain variable in size among members of the tetraspanin family [18, 20].

The presence of an energetically unfavorable hydrophobic patch and the dimeric form of CD81 LEL in a crystal hinted at the high likelihood of tetraspanin assembly into dimers or multimers in the cell membrane. As shown later, clustering of tetraspanins leads to the organization of tetraspanin-associated proteins in a tetraspanin web or tetraspanin-enriched microdomain (TEM) [24, 25]. Biologically, such domains induce partitioning of the complexes into lipid rafts and clustering of the lipid rafts [26].

Apart from the hydrophobic interactions of the extracellular domains, the membrane-proximal cysteines residing in transmembrane domains contribute to the dimer formation and stability of tetraspanins. For the CD9 tetraspanin, it has been demonstrated that these residues are positioned close to the dimerization interface and influence homotypic and heterotypic tetraspanin association depending on their reversible palmitoylation status [27]. Similarly, the mutation of N-terminal

and C-terminal transmembrane cysteines to serine eliminated palmitoylation of CD151, which turned out to be deleterious for the assembly with other cell surface proteins, including tetraspanins CD9 and CD63, their organization to TEMs and subsequently their subcellular distribution and cell morphology [28]. At the same time, it had minimal influence on the density of tetraspanin protein complexes and was dispensable for CD151-$\alpha_3\beta_1$ integrin association. Depalmitoylation of CD81 did not impact its surface expression and stability, but rendered it less available for contact with its natural interaction partner CD9 and the relevant epitopes less accessible for binding of structurally dependent antibodies [29].

The four transmembrane helices of tetraspanin proteins form two largely separated pairs of antiparallel helices: one pair comprises TM1/TM2 and the other TM3/TM4. The two pairs of helices only converge close to the cytoplasmic side of the membrane through contacts between TM2 and TM3. In the recently solved crystal structure of full-length CD81, this cone-like structure has been shown to harbor a binding pocket for cholesterol [30], and mutations within transmembrane domains in certain tetraspanins have been connected with pathological states [31]. The short cytoplasmic tails show no obvious functional significance in signaling processes, suggesting that their signaling competence relies on association with other molecules [32]; nevertheless, its mutation can lead to different assembly with association partners as shown for CD9 [33].

3. Natural ligands of tetraspanin proteins

3.1 The tetraspanin web and intertetraspanin contacts

The ability of members of the tetraspanin family to assemble into a unique biological feature known as tetraspanin-enriched microdomain (TEM) is due to their mutual interactions; however, these structures include also receptors, integrins and signaling molecules such as phosphatidyl-kinase C (PKC) and phosphatidylinositol-4-kinase (PI4K) [9]. These interactions are fundamental for cellular functions such as cell adhesion, proliferation and motility. Interactions between tetraspanin members are important in maintaining the integrity and stability of the tetraspanin web and providing binding sites for different ligands. The multimers of newly synthesized proteins are formed in the Golgi apparatus. The predominantly cross-linked tetraspanin species are homodimers, but also higher order complexes and low amounts of heterodimeric tetraspanins (CD81/CD9, CD9/CD151, CD81/CD151) were identified [27]. It has been suggested that tetraspanin homodimers, formed in the Golgi and present at the cell surface, serve as building blocks in the assembly of higher organized tetraspanin protein complexes. Interestingly, the exosomes originating from cell lines overexpressing CD9 are believed to be enriched in more stable TEMs [10]. Overall, although most tetraspanins can interact with most other tetraspanins, and similarly engage with several other proteins, the nature of these interactions has been until recently classified only according to their stability in the presence of detergents of different stringency, which does not necessarily reflect their significance in the cellular milieu [34]. A thorough characterization of strength and abundance of the interactions between the members participating in a tetraspanin web in a particular cell and physiological situation is therefore needed and will support the understanding of its mediated biological effects. Similarly, most data on tetraspanin functionality come from studies on their localization on cell membranes, while functional data in vesicles are still scarce. Therefore, we here summarize the known cellular functions, while speculating how this might translate to EVs.

An important step towards the understanding of the specificity of the tetraspanin interactions in TEM has been achieved by delineation of the involved tetraspanin regions by dissecting the model tetraspanin into domains, differently amenable for modification. Early experiments that addressed the relatively unstructured and at the same time antigen-binding competent regions that appeared attractive for mutagenesis resulted in a protein that showed aberrant clustering involving both homo- and heterodimerization of resulting full-length tetraspanins [35], albeit the mutagenesis method employed in this study was a complete deletion of targeted domains. The CD81 D-region was studied in more detail: the CD9 and CD151 tetraspanins were more competent of clustering with CD81 when homologously engrafted with CD81 D-region [36]. When the mutagenized CD81 EC2 molecular subunits were transplanted to other tetraspanins, the extremely flexible conformation of the solvent-exposed D-segment of CD81 EC2 was sufficient to overcome the orientational restrictions to initiate the homotypic contact for dimerization, and this finding has been corroborated with both wet-lab data and the insights from molecular dynamic simulation of the cell membrane-embedded protein [37].

3.2 Interaction of tetraspanins with integrins and matrix-degrading enzymes: role of tetraspanins in cancer and metastasis

Important association partners of tetraspanins are the integrins. The role of such complexes in invasive growth *in vivo* as well as the effect of integrin-mediated binding events on cell proliferation and invasion is well established. Especially, the laminin-binding integrins ($\alpha_6\beta_4$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_7\beta_1$) exhibit extensive interactions with tetraspanin proteins [12, 38]. The functionality of integrins may depend critically on their interaction of tetraspanins: it has early been described that the remarkably stable association of the tetraspanin CD151 and the integrin $\alpha_3\beta_1$ leads to a high level of activation of cellular PI4K [39]. Further, CD151 interacts directly with the α_3 subunit and links it to other tetraspanins, CD9 and CD81. Loss of CD151 abrogates the $\alpha_3\beta_1$ mediated mobility on its ligands, laminin-332 and laminin-551. CD9/CD81 complex may even regulate the integrin-mediated functions independently of CD151 by forming a complex with the integrin and directing the PKCα-$\alpha_3\beta_1$ association [40]. Another example of tetraspanin-integrin association reveals its proangiogenic role through VEGF induction, mediated by cooperation between TM4SF5 and integrin α_5 of epithelial cells [41]. Interestingly, removal of CD151 palmitoylation sites did not disrupt the CD151–$\alpha_6\beta_4$ complex in epithelial cells but strongly influenced $\alpha_6\beta_4$-integrin–dependent cell morphology [42]. The rat tetraspanin D6.1A (human homolog is CO-029) was able to induce systemic angiogenesis by initiation of an angiogenic loop that reached organs distant from the tumor, probably due to the abundance of D6.1A in tumor-derived exosomes. This is in line with reports claiming that EVs prepare niches for metastatic tumor cells at tissues distant from the primary tumor [43]. This tetraspanin associates with integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$, as well as with tetraspanins CD9 and CD81, and is similarly to CD151 linked to tumor-promoting activities [44].

Active complexes of tetraspanins and integrins influence biological processes other than cellular signaling by interacting with cellular metalloproteinases, important players in the remodeling of extracellular matrix. A study of MDA-MB-231 cells, a breast cancer cell line, has indicated that the $\alpha_3\beta_1$-tetraspanin protein complex may be linked to an invasive phenotype of tumor cells via modulation of various signaling pathways, including activation of membrane metalloproteinase-2 (MMP-2), an enzyme associated with invasive migration of the cells, and affecting phosphatidylinositol-3-kinase (PI3K) signaling pathways, which control actin cytoskeleton dynamics [45]. By the incorporation of the members of a disintegrin and metalloproteinase (ADAM) family members the tetraspanins are able to influence the

cellular ectodomain cleavage and release activity of these enzymes [46]. The tetraspanins of the TspanC8 group (tetraspanins with 8 cysteines) have a significant impact on the cellular exit and catalytic activity of ADAM10 [47], in particular the activity of Tspan15/ADAM10 promoted N-cadherin cleavage [48–50]. Different TspanC8/ADAM10 complexes seem to have different substrate specificities [51]. The silencing of CD9 enhanced shedding of ADAM17-substrates TNF-α and ICAM-1 [52].

Important discovery of the possible consequences of fine differences in composition of TEMs has been delivered by the study of exosomes enriched in Tspan8-α_4 complex that were preferentially taken up by the endothelial and pancreatic cells [53]. The fact that such modifications can allow selective targeting *in vitro* and *in vivo* holds promise to achieve improved exosomal delivery by engineering of their membrane components.

3.3 The role of tetraspanins in immune complexes

In antigen-presenting cells (APCs), tetraspanins integrate into TEMs protein-recognition receptors binding to conserved repeated motifs of microbes, such as Toll-like receptors, and MHCII molecules into tetraspanin web platforms, as well as Fc_γ receptor I in phagocytic cells, Fc_γ receptor IIb and III upon the activation of macrophages and Fc_ε receptor I in monocytes and skin-derived dendritic cells [54].

The particular role of CD81 protein in the formation of specialized microdomains in the plasma membrane of the cells of the immune system was discovered by elucidating its function of recruiting various adhesion molecules, receptors and signaling proteins to the central zone of the immune synapse in T-lymphocytes and APCs [55]. Therefore, it has early been proposed for CD81 to play a key role during antigenic presentation, since it colocalizes with the T-cell receptor/CD3 [56], and CD81 indeed turned out to be a regulator of CD3 clustering and sustained CD3 signaling [57].

Further, the T-cell side of the immune synapse is densely populated by tetraspanins CD9 and CD151. The abolishment of their expression reduces markers of activation of T-lymphocytes conjugated to the APCs, such as IL-2 secretion and expression of CD69 [58].

Another role in the immune response of tetraspanin CD81 is amplifying and sustaining B-cell receptor signaling from lipid rafts by ligation to the co-receptor CD19/CD21 complex. The signaling through a variety of cell surface protein complexes implies a role of lipid rafts, again highlighting the ability of tetraspanin to facilitate raft association [9].

3.4 Tetraspanins in pathogen infection

Several studies have been oriented towards the research of tetraspanins as ligand molecules for pathogen entry. CD81 has been identified as a ligand for hepatitis C virus (HCV) recognition and viral entry [59]. The ligand for viral glycoprotein E2 is the D-domain of the LEL, a dynamic region positioned within the triple-bundle helix, whose conformation in solution differs substantially from the one suggested by crystal structure. Challenging for structure-based design, this region nevertheless presents an attractive target for design of therapeutically relevant ligands with methods such as NMR [60]. Apart of the EC2 domain, other regions of CD81 have proven important for virus infection. Experimental evidence here was based on the exchange of the structural domains of the molecule with the ones of tetraspanins of different degrees of homology, and it was found that closely related substitutions were more efficient at functional complementation of CD81. Viral entry has been shown to correlate with surface expression of the chimeric protein and to depend on the presence of the cholesterol-coordinating glutamate residue [61]. EWI-2wint,

a cleavage form of EWI-2, a member of the immunoglobulin superfamily, has an inhibitory effect on HCV infection by obstructing the interaction between CD81 and HCV E2 [62]. The related factor EWI-F inhibits *Plasmodium* infection, whereas its silencing increases infection efficiency [63].

Tetraspanin microdomains have been described to regulate HIV-1 entry, assembly and transfer between the cells [64, 65]. CD81 influences importantly the early stages of virus replication by controlling the stability of HIV-1 restriction factor and consequently the activity of viral reverse transcriptase [66], while CD63 facilitates endocytosis of the HIV receptor CXCR4 [67] as well as supports the replication steps in macrophages [68, 69]. Also Coronaviruses and low-pathogenicity Influenza A viruses utilize TEM domains as entry portals to co-engage with cellular receptors and proteases, which enable viral proteolytic priming [70]. As shown in an *in vivo* model with CD151 null-mice, this tetraspanin is a critical novel host factor of nuclear export signaling of Influenza A virus, used complementary to the viral nuclear export proteins [71].

Tspan9 modulates the early endosome compartment to make it more permissive for membrane fusion of early-penetrating viruses, and its depletion strongly inhibits infection by alphaviruses that fuse in early endosomes but does not alter the delivery of virus to early endosomes or change their pH or protease activity [72]. It is unclear, what function then EV-based tetraspanins might have in the context of viral infection, and it might be speculated that cells use EVs to titrate away virus into membrane structures that are unable to provide replication and protein synthesis machineries for the virus. This is supported by the findings that EVs might have anti-influenza infection activity *in vitro* [73].

4. Extracellular vesicles as mediators of cell-cell interaction

4.1 Biological basis for therapeutic applications: EVs as mediators of intercellular interactions

EVs are secreted by most cell types and are taken up by recipient cells, where their cargo consisting of a cocktail of proteins, mRNAs and non-coding RNAs alters the behavior of the recipient cells in a way that might be even considered similar to hormones or cytokines [74], e.g. in the context of skin or bone cell paracrine signaling [75–77]. siRNAs (small interfering RNAs) and miRNA-based inhibitors have been recognized as potent novel drug candidates for many years. As EVs can be loaded with different drugs *in vitro*, they qualify as an attractive drug delivery system. The specificity of the recipient cell targeting *in vivo* is understood in a limited way only, although there is evidence of accumulation of specific EVs [43]. For example, EVs from human mesenchymal stem cells accumulated in the liver, spleen and sites of acute kidney injury [78]. Such tropism for a specific cell type, a requirement for targeted drug delivery, appears to be determined by surface proteins of the source cells. The composition of EV membrane reflecting the one of their source cell makes these particles non-immunogenic, and their small size allows them to pass the immune surveillance of the host organism [79, 80]. The reported engagement of exosomes in physiological processes in normal and diseased central nervous system makes them attractive vehicles for delivering neurotherapeutic agents across the blood-brain barrier [81–83]. Nevertheless, their delivery in humans seems so far limited to liver and kidney as they are reported not to reach therapeutic amounts in brain, heart and other tissues due to lack of specific targeting and thus low enrichment of the intended therapeutic ingredient in the target tissue. Modifications of the EV surface membrane to achieve enhanced targeting of a specific cell type are hence a common strategy embodied in several different engineering approaches.

4.2 Mechanisms of EV entry into the target cell

When an EV attaches to the target cell surface, it can in some cases activate the cognate receptors without internalization or transfer of the content to the recipient cell via its fusion with the target cell membrane or via endocytosis [84]. Endocytosis is an active process that requires cytoskeletal remodeling dependent on actin dynamics and includes clathrin-dependent endocytosis, phagocytosis and macropinocytosis. The clathrin-dependent endocytosis has been established as cellular entry for EVs based on the experiments with specific inhibitors of this pathway. Additionally, endocytic uptake of EVs can involve lipid rafts, sometimes dependent on caveolin proteins. The size of EVs may be a limiting factor for cellular entry via endocytosis [85]. The EV uptake by phagocytosis was monitored by their high level of accumulation in phagocytic cells and localization into the phagolysosome [86], as well as the identification of the crucial role of the phosphatidylserine binding T-cell immunoglobulin and mucin domain containing (TIM4) receptor for the uptake of exosomes into macrophages [87, 88]. The contribution of macropinocytosis pathway was revealed with studies where exosomal uptake was decreased by the inhibition of cytoskeletal rearrangements that normally lead to membrane ruffles [89], as well as with its promotion caused by the activation of the agonistically acting epidermal growth factor [90].

5. Molecular engineering to facilitate EV labeling and delivery to target cells

5.1 Modifications of EV membrane with non-covalent and chemical modifications

The role of EVs as encapsulated intercellular messengers makes them attractive for development into nanoscale therapeutic agents [91], and therefore, the need of augmenting the interaction with the recipient cell has been widely recognized. The higher rigidity of the membrane of EVs in comparison with their source cells does not appear to obstruct the efficient application of common hydrophobic insertion strategies to EVs. The introduction of small lipophilic ligands, such as membrane dyes, works effectively for their labeling and aids in monitoring in *in vitro* and *in vivo* experiments. Furthermore, hydrophobic loading is used for encapsulation of certain drugs and leads to their increased stability and therapeutic effect [92, 93].

Due to the negative charge on the recipient cells, binding and uptake of EVs were enhanced by increasing the charge interactions with an artificially introduced positive surface potential, as exemplified in their derivatization with cationic lipids [94]. A downside of this method can be that the extremely charged cationic reagents can cause cytotoxicity and the cellular uptake of the modified particles can proceed differently from the usual pathways, which results in an unpredictable cellular fate of the particle and its cargo and possibly its undesired degradation. The innate slightly negative electrostatic potential exhibited by unmodified EVs should contribute to the longer half-life of such particles *in vivo* as inferred from liposomal studies [95]. Further, the extremes of unbalanced positive charge may negatively impact the storage stability and the application of such reagents in high concentrations.

As an alternative to other methods, labeling of EV surfaces with fluorescent probes has been achieved using click chemistry without an apparent effect on the size and function of the particles [96]. Nevertheless, there is an immense complexity behind the engineering and downstream methods developed for other

biologicals conjugated with small molecules, especially when intended for therapeutic purposes [97, 98].

The idea of decoration of EV membranes with functional ligands further led to the consideration of receptor binding strategies. Such EV functionalization can readily be achieved by modification of the source cell. A resulting opportunity harvesting the specificity of this approach could also be used as a strategy to eliminate vesicles implicated in pathological processes, such as cancer metastasis, or to neutralize the undesired activity of therapeutically applied vesicles. Transferrin-conjugated superparamagnetic nanoparticles, reactive with surface-expressed transferrin receptor of exosomes, enabled their isolation from blood and endowed the vesicles with superior targeting properties [99]. A robust labeling approach of microvesicles was the expression of biotin acceptor peptide-transmembrane domain (BAP-TM) receptor on the source cells in combination with biotinylation *in vivo* [100].

Another example of employing an EV-localizing protein for efficient presentation on the EV surface involved a peptide or a protein fused with the C1C2 domain of lactadherin, which binds to EV outer membrane due to its affinity to phosphatidylserine, strongly enriched in exosome membranes. This method was used to generate antibodies against tumor biomarkers [101] and to increase the host immune response to tumor-associated antigens [102, 103].

Strong binding of lactadherin to the exosome membrane was also the basis of an efficient labeling protocol utilizing overexpression of a fusion protein composed of *Gaussia* luciferase and a truncated lactadherin in source melanoma cells. After harvest by ultracentrifugation, the labeled exosomes were successfully used for *in vivo* biodistribution studies [104].

5.2 Engineering of tetraspanin proteins for detection and monitoring of EVs

Regarding the tetraspanins not only as very abundant, but as prominent structural and stability elements of the exosomal membrane, it is sensible to engineer genetic fusions of these proteins enriched in EVs to ensure a high density of the expressed product and optimize the derivatization for the different purposes of engineering: cognate ligand binding, labeling for visualization or isolation, modifying cargo uptake and transfer and stability (**Figure 3**). Most practically, transgene

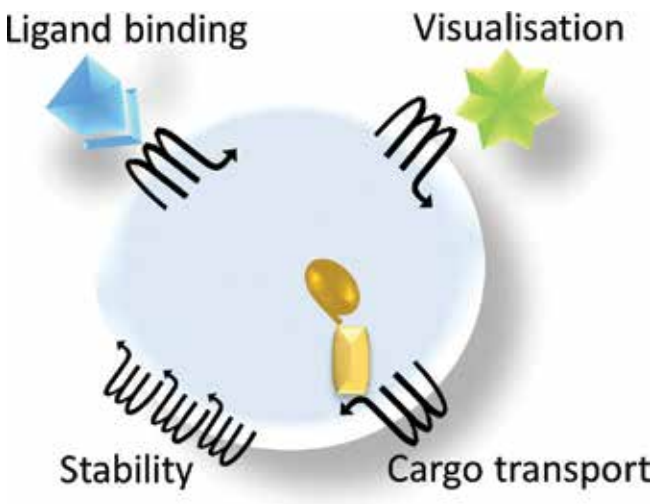

Figure 3.
Main engineering goals utilizing the modification of tetraspanins.

expression in the parent cell would be induced to deliver EVs enriched in the modified protein. The success of such set-up depends on the understanding of both molecular biology and protein architecture of the targeted species.

In one of the pioneering studies, certain sites on the tetraspanin CD63 have been chosen to allow the integration of fluorescent fusion proteins on the extra- and intravesicular side of the exosomal membrane [105]. CD63-GFP fusions have proven valuable reporters in the elucidation of the role of immune synapse in secretion of exosomes from T-cells to APCs and their fusion with recipient cells [106], in determining differential uptake properties of different immune cell subsets for EVs originating from a cancer cell line [107] and in an *in vivo* study imaging the fate of EVs produced by breast cancer cell line in *nude* mice [108].

5.3 Engineering of EV surface proteins for enhanced cell-type specific targeting: introduction of target specificity, stability and improved cargo delivery

In the pioneering example of derivatization of a protein enriched in exosomal membrane to achieve specific targeting [109], the rabies virus glycoprotein (RVG)-peptide fused with lysosome-associated membrane glycoprotein 2b (Lamp-2b) was used to target exosomes to the central nervous system in an *in vivo* mouse model. Immature dendritic cells-derived exosomes, enriched in an N-terminal fusion of an α_v integrin-specific internalizing RGD-sequence containing peptide with this scaffold, could internalize efficiently into target-positive breast cancer cells [110]. The display of such constructs could be efficiently enhanced by introducing a protective glycosylation motive, which improved the surface expression of exosome-bound N-terminally fused peptides by preventing their acid-mediated proteolysis during endosomal passage and indeed led to a more efficient specific cellular uptake of exosomes engineered in this way [111]. Specific targeting of IL3-receptor overexpressing chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells has been shown for exosomes armed with a fusion protein of IL3 and Lamp2b and loaded with Imatinib, a tyrosine kinase inhibitor, and led to a the reduction of tumor size *in vivo* [112]. Exosomes engineered in this way achieved a higher abundance at the tumor site and were hence able to inhibit xenograft growth more efficiently than the active ingredient alone or than loaded control exosomes.

Glycosylphosphatidylinositol (GPI)-mediated anchoring of the specific targeting unit was demonstrated to be a stable alternative to Lamp2b fusions. EVs expressing GPI-anchored nanobodies, specific to epidermal growth factor receptor (EGFR), displayed enhanced binding to the EGFR-overexpressing cancer cells. This, however, did not lead to an increased uptake, and it was suggested that in this particular biological system not only the affinity but also the density of the targeting ligand must be high enough to induce receptor clustering and subsequent internalization [113]. Exosomes derived from a HEK cell line transfected with a construct of platelet-derived growth factor (PDGF)-anchoring sequence and an EGFR-binding peptide were on the other hand efficient in targeting EGFR-expressing tumors *in vivo* and reducing their size with delivery of microRNA Let7 [114]. Enhanced uptake has been achieved for EVs enriched in a fusion protein of tetraspanin CD63 and stearylated octaarginine, a representative cell-penetrating peptide, by their ability to induce active macropinocytosis [115].

The concept of enhancing the stability of the targeted exosomal surface protein intended for fusion with a targeting agent to allow a higher degree of versatility for their modification for an improved target recognition has raised interest in their engineering at the protein level. A significant increase in thermal stability has been achieved by introduction of additional disulfide bonds in the EC2 of tetraspanin CD81, with the best variant exhibiting a positive shift in the melting temperature

for 45°C comparing with the wild-type protein [116]. When engrafted with a human transferrin-receptor specific peptide, the stabilized scaffold exhibited significantly better biophysical properties than the analogously engrafted wild-type protein. In the same study, a mutant has been discovered that exhibits reversible unfolding behavior up to a temperature of 110°C in contrast to the wild-type CD81 EC2, which presents another option for extensive engineering required for directed evolution of tetraspanin proteins towards novel antigen binding.

Not only can the overexpression of tetraspanins in source cell lines increase the production and stability of exosomes, but also the exosomes engineered to contain adeno-associated viruses (AAVs), designed for improved delivery of genetic material to target cell [100], were superior in their yield and functionality when CD9 was overexpressed in AAV-producer cells [10]. CD9 overexpression has also increased the speed and transduction efficiency of lentiviral gene delivery into numerous cell lines, confirming the important role of this tetraspanin in gene transfer [117].

The current scope of EV engineering reaches beyond receptor targeting systems and aspires towards modifications with complex modules, assigning them simultaneously with multiple novel functionalities, such as specific recognition as well as enzymatic activity, to enhance their potential therapeutic effect. Recently, regression of orthotopic Her2-positive tumors has been achieved by applying exosomes, able of specific targeting via their surface decoration with a fusion protein of high-affinity anti-Her2 single-chain Fv, and containing mRNA encoding a bacterial enzyme Hchr6, which in the strongly Her2-positive cells catalyzed the conversion of the prodrug CNOB to cytotoxic MCHB [118]. Tetraspanin engineering could support a sophisticated concept to aid intracellular delivery of exosomal cargo proteins, directionally incorporated during vesicle biogenesis [119]. Genes encoding two recombinant proteins, a fusion of photoreceptor cryptochrome 2 (CRY2) and the protein of interest and a fusion of tetraspanin protein CD9 and CRY-interacting basic-helix–loop–helix 1 (CIB1), were co-transfected into a single cell line. The blue light-induced binding of CRY2 and CIB1 enabled docking of CRY2-fused target proteins into nascent exosomes, and in the absence of the blue light, the cargo protein was released into the exosomal lumen. Transfer of Cre recombinase confirmed the efficiency of this system *in vitro* and *in vivo*.

6. Conclusions

The tetraspanins, well established as the biomarker proteins of extracellular vesicles, have been addressed for increasing EV stability and improving their function as delivery vehicles, both by assigning them with target recognition properties and modulating their cargo transfer. From the current point of view, the complexity of the tetraspanin-mediated interactions and signaling networks formed in a cell is yet to be discerned. Tetraspanins are known to interact naturally with a plethora of cell surface-bound ligands, which results in potent biological effects conveyed through different pathways; however, systematic evaluation of the affinity of the association with the interaction partners would assist in prediction of the consequential cellular processes as well as in determining optimal choice of the tetraspanin targeted for modification. There are recent reports describing modified tetraspanins mediating both surface protein interactions and an intracellular fusion-mediated enzymatic activity, which underline the feasibility of engineering versatile functions into tetraspanin proteins as fusion partners. The structural details on tetraspanins modified in this way, as well as the read-out revealing their actual behavior in the foreseen role and the influence of such modifications on

the fate of an EV preparation, will pave the way into the design and production of EV-based reagents as therapeutics.

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Conflict of interest

The authors declare no conflict of interest.

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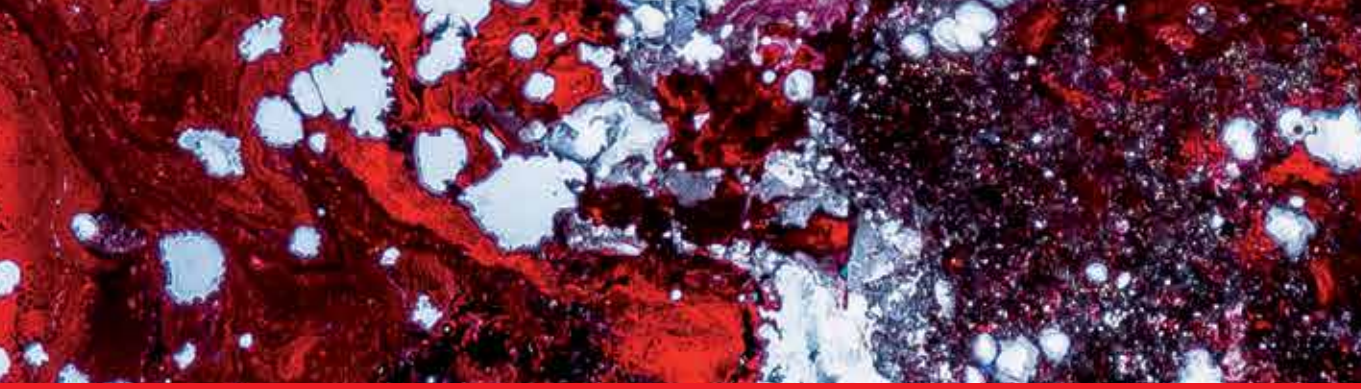

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Genetic and cellular technologies in life science have recently achieved remarkable progress, and thus the roles of biochemical engineers have also been changed to incorporate the use of new technology. Therefore, this book deals with current topics in biochemical engineering. The chapters of this book discuss research that has introduced artificial enzymes, kinetic models in bioprocessing, a small-scale production process, and production of energy with microbial fuel. These chapters offer novel ideas for the production of effective compounds and energy. Moreover, other research has introduced the production technology of stem cells and biomedical processes using nanoshells and extracellular vesicles. These chapters will provide novel ideas to produce effective compounds and develop therapies for various diseases.

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